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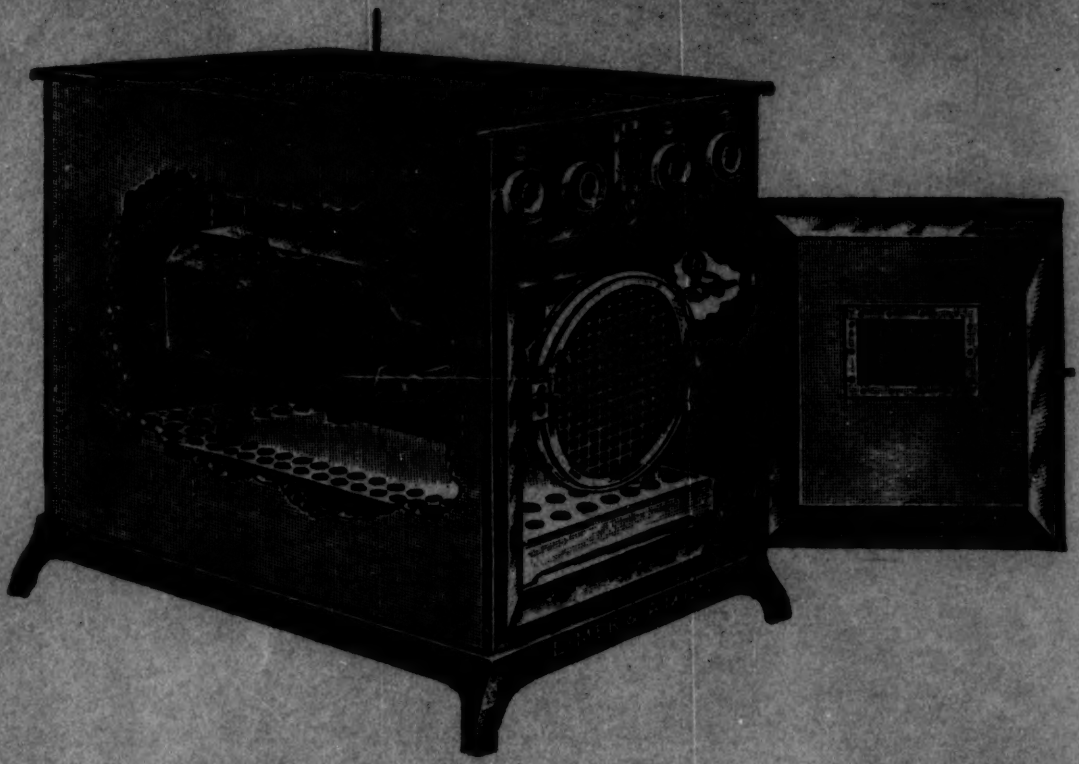

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THE OCCURRENCE AND DETERMINATION OF CREATINE IN THE URINE.

By F. H. McCRUDDEN and C. S. SARGENT.

(*From the Laboratories of the Robert B. Brigham Hospital, Boston.*)

(Received for publication, February 15, 1916.)

Some months ago we began to find creatine with surprising frequency in the urine of different patients, some of whom had shown no creatine on previous occasions. The fact that in all cases in which creatine was found the determinations were made by Folin's new method[1]—conversion of the creatine to creatinine by boiling with picric acid—whereas the earlier determinations had been made by the old method—in which the conversion is brought about by boiling with hydrochloric acid—made us suspect the method. Various control experiments were, therefore, made in an attempt to clear up the difficulty, the result of which is that we have been forced to conclude that the new method is not reliable; and that something other than creatine which gives the creatine reaction occurs in almost every human urine.

Experiments with Urine Alone.

1. A woman, slightly incapacitated with hypertrophic arthritis but otherwise well, was put on a diet free from meat, fish, soup, peas, beans, tea, and coffee, and containing chiefly eggs, milk, bread, rice, vegetables, and salads. The urine was found to contain 1.019 gm. creatinine in 24 hours.

3 cc. of urine, from a 24 hour amount of 3,000 cc., were treated with 20 cc. of a saturated solution of picric acid and 1.5 cc. of a 10 per cent sodium hydroxide solution and allowed to stand 10 minutes before being compared in a Duboscq colorimeter with a standard creatinine solution. The standard was 1 cc. of a 0.1 per cent solution of pure creatinine to which 20 cc. of a saturated solution of picric acid and 1.5 cc. of a 10 per cent solution of

[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 469.

sodium hydroxide had been added; this was allowed to stand 10 minutes before being compared with the unknown. In these and the following determinations the standard was set at 20.0.

The readings of the unknown were as follows:

	1.	2.
	20.1	19.4
	19.3	19.2
	20.2	19.2
	20.4	19.8
	19.9	19.9
	18.6	19.5
Average,	19.75	19.5

These, when calculated for the total urine, give 1.012 and 1.026 gm. creatinine, an average of 1.019 gm.

2. The sum of the creatinine plus creatine in this urine was then determined by Folin's older method. It was found to be the same as the amount of creatinine alone, 1.020 gm. Creatine was, therefore, absent.

30 cc. of urine and 15 cc. of approximately N hydrochloric acid were heated on the water bath for 3 hours, cooled, and diluted in a volumetric flask to 50 cc. 5 cc. of this mixture—equivalent to 3 cc. of urine—were taken for the colorimetric determination; this was neutralized with sodium hydroxide; picric acid and alkali were added, and the colorimetric determination was carried out in the same way as before.

Duplicate determinations gave the following readings:

	1.	2.
	19.8	20.2
	19.0	19.6
	18.8	19.8
	19.8	19.5
	19.5	20.2
	19.3	19.4
Average,	19.4	19.8

These show respectively 1.030 and 1.010 gm. creatinine, an average of 1.020.

3. The sum of creatinine plus creatine was then determined by the new method. The amount found was 1.133 gm. According to this method the urine contains, therefore, 1.133 minus 1.019 or 0.114 gm. creatine.

To 3 cc. of urine were added 20 cc. of a saturated solution of picric acid and 130 cc. of water; the solution was boiled gently for 1 hour, after which

it was rapidly boiled down to 20 cc., then cooled, and 1.5 cc. of a 10 per cent sodium hydroxide solution were added to it. After 10 minutes the color was compared with that of the standard.

The readings were:

	1.	2.
	17.6	17.4
	18.2	17.5
	18.0	17.5
	17.8	17.6
	17.8	18.3
	17.6	17.0
Average,	17.8	17.55

These give 1.126 and 1.140 gm., respectively, of creatinine plus creatine (average 1.133 gm.).

4. A determination of the creatine by the new method was then made on the specimen which had been boiled with hydrochloric acid for determination of creatine by the old method (see 2); this determination gives, therefore, the results found by superimposing the new method on the old method. The amount found was 1.158 gm. or 1.158 minus 1.019, equal to 0.139 gm. of creatine, practically the same as found by the new method alone.

5 cc. of the mixture used in 2 were boiled for 1 hour with 20 cc. of a saturated solution of picric acid and 130 cc. of water, then evaporated quickly to 20 cc. and cooled; 1.5 cc. of a 10 per cent sodium hydroxide solution were added and the color obtained was compared with that of the standard.

Duplicate determinations were made and the average of six readings was taken in each case as before.[2] The average readings were 17.05 and 17.55, respectively. This gives 1.173 and 1.143 gm. creatinine plus creatine, an average of 1.158.

These figures are but examples of those obtained in numerous analyses, the limits of error of which cannot be more than 0.02 to 0.03 gm. creatinine. This seems to indicate that the results obtained by the new method are not due simply to a more complete conversion of creatine into creatinine by the new method, but to the presence in the urine of some compound other than creatine which gives a color reaction on boiling with picric acid, but not in the cold; and which does not change to creatinine on boiling with hydrochloric acid.

[2] In these and all the following experiments at least six readings were taken in every case, just as in 1, 2, and 3.

Experiments with Pure Solutions.

On showing our results to Dr. Folin he very generously offered us, for the purpose of testing the matter further, the specially purified creatine and creatinine prepared in his own laboratory; and in the following experiments these were used.

Analyses 5, 6, and 7 were carried out to determine whether pure creatine reacts differently by the two methods, the old and the new; and whether creatinine itself gives higher results on boiling with picric acid than it does in the cold.

5. Two 1 cc. specimens of a 0.1 per cent solution of pure creatinine were boiled for 1 hour with picric acid as in the new method. Comparison with the standard showed an average of 1.029 mg. creatinine,[3] an amount which is within the limits of error.

6. To this same 0.1 per cent solution of creatinine enough pure creatine was added to make the solution contain 0.015 per cent creatine (equivalent to 0.013 per cent creatinine). Analyses of the solution by the old method showed 1.157 mg. creatinine plus creatine, or 1.157 minus 1.029, equal to 0.128 mg. creatine in 1 cc. (instead of the 0.130 mg. present).

7. Experiment 6 was repeated using the new method instead of the old method for converting creatine into creatinine. The result was the same, 0.128 mg. creatine.

The results show that creatinine itself does not give higher results on boiling with picric acid than it does in the cold; and that creatine is converted quantitatively into creatinine on boiling with hydrochloric acid. The inference drawn from the earlier experiments, namely, that the deepening of the color on boiling urine with picric acid and then adding sodium hydroxide, is due to something other than creatine, is confirmed by these experiments.

Experiments with Pure Solutions plus Urine.

In order to see if conditions in the urine alter the behavior of creatine, experiments similar to the preceding three were repeated on urine to which pure creatine had been added. For purposes of description we will refer to real creatine here as *a*-creatine and

[3] In these and the succeeding experiments duplicate determinations were always made; the figures given show the average result.

to the supposed new substance or substances responsible for the reaction on boiling with picric acid as *x*-creatine; we do this merely for convenience and do not thereby mean to imply that *x*-creatine has any chemical relationship to *a*-creatine or even that it is a single substance. The urine used in these experiments was the same as that used in Experiments 1 to 4; it contained 1.019 mg. creatinine, no *a*-creatine, and 0.138 mg. *x*-creatine (calculated as creatinine) in 3 cc.

8. 0.150 mg. creatine was added to 3 cc. of the urine and the creatinine plus creatine determined by the old method. The amount found was 1.163 mg. This gives 1.163 minus 1.019, equal to 0.144 mg. creatine.

9. In the same solution of creatine in urine used in the preceding experiment, creatinine plus creatine was determined by the new method. The amount found was 1.282 mg. Subtracting from this the creatinine plus *x*-creatine present, as found in Experiment 3, we get 0.124 mg. of *a*-creatine (calculated as creatinine) which is practically the amount added (0.130 mg.).

The results show that the conditions occurring in the urine do not affect the behavior of creatine in such a way as to account for the results.

Experiments with Other Reducing Substances.

The possibility that the results might be due to the presence of other reducing substances led us to try the effect of small amounts of glucose and uric acid on the reaction.[4]

The urine used in these experiments was free from any reducing substances detectable by the ordinary methods; but since glucose does give a color reaction on boiling with alkaline picrate we tried the effect of such quantities of glucose and uric acid on the reaction as might occur in normal urine.

A 300 cc. specimen of urine was divided into three portions, *a*, *b*, *c*, of exactly 100 cc. each. To *a* nothing was added; to *b* 30 mg. of pure glucose; to *c* 30 mg. pure uric acid. Creatinine, *a*-creatine, and *x*-creatine were then determined on all three specimens.

[4] The statements of Folin (*J. Biol. Chem.*, 1914, xvii, 473) and S R. Benedict (*J. Biol. Chem.*, 1914, xviii, 191) regarding the effect of sugar on the reaction are somewhat conflicting.

The amount of creatinine found, calculated in mg. per 100 cc., was as follows:

	a	*b*	*c*
Creatinine	2.29	2.28	2.28
Creatinine plus creatine (boiling with hydrochloric acid)	2.39	2.41	2.35
Creatinine plus creatine (boiling with picric acid)	2.58	2.55	2.59

This urine—not the same as that used in the previous experiments—contains 0.10 mg. *a*-creatine and 0.20 mg. *x*-creatine. The results are not affected in the slightest degree by the glucose and uric acid added.

DISCUSSION.

The figures show conclusively that there is something in the urine other than creatine which, on boiling with picric acid, gives a color reaction similar to the creatinine reaction. The results cannot be accounted for by assuming that the creatinine itself gives a deeper color on boiling with picric acid than it does in the cold (Experiment 5); they are not due simply to a more complete change of the creatine into creatinine on boiling with picric acid than on boiling with hydrochloric acid (Experiments 6 and 7); they are not due to a partial destruction of the creatine on boiling with hydrochloric acid (Experiment 4).

Since the new method shows creatine in amounts of 0.1 to 0.2 gm. per day in almost every urine, whereas the old method shows no creatine in normal urine, but does show it in the urine in cases of various muscular dystrophies, starvation, diabetes, and other conditions where we might expect it to be found, we can say that in the present state of our knowledge of creatinine and creatine metabolism, the results obtained by the old hydrochloric acid method are probably more significant than those obtained by the new picric acid method.

The amount of *x*-creatine found is variable; we have not observed that it shows any relationship to the patient's weight or to the diet. The following table shows the amounts of creatinine, *a*-creatine, and *x*-creatine in gm. per day found in two nearly normal patients. The table gives fair examples of the amounts of *x*-creatine ordinarily found; it is occasionally absent from the urine.

Day.	Woman.			Man.		
	Creatinine.	α-Creatine.	x-Creatine.	Creatinine.	α-Creatine.	x-Creatine.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*
1	0.651	0	0.130	1.924	0	0.161
2	0.680	0	0.107	1.929	0	0.402
3	0.653	0	0.141	1.975	0	0.190
4	0.655	0	0.073	1.989	0	0.102
5	0.650	0	0.173	1.970	0	0.209
6	0.633	0	0.167	1.964	0	0.154

The following table shows the figures obtained in a case of progressive muscular dystrophy where real creatine was present as well.

Day.	Creatinine.	α-Creatine.	x-Creatine.
	gm.	*gm.*	*gm.*
1	1.483	0.283	0.012
2	1.503	0.316	0.091
3	1.481	0.599	0.072
4	1.493	0.624	0.007
5	1.486	0.578	0.060
6	1.481	0.409	0.110
7	1.471	0.448	0.068

These results were obtained several months ago. We had then hoped to find time to isolate the substance or substances which we have called x-creatine. The necessity of postponing further work on the subject for some time to come makes it seem advisable to publish our findings in order to call to the attention of other workers the inaccurate conclusions which might be drawn on finding so called creatine in the urine by this method.

SUMMARY.

Human urine contains a substance or substances other than creatine which can give a color reaction similar to that of creatinine on boiling with picric acid, and which, therefore, may appear in the results as creatine.

A CRITIQUE OF CERTAIN DATA ON THE CONTENT OF CHOLESTEROL AND FATTY SUBSTANCES IN THE BLOOD, TOGETHER WITH A MODIFICATION OF THE COLORIMETRIC METHOD FOR ESTIMATING CHOLESTEROL.

By FRANK A. CSONKA.

(*From the Laboratory of Dr. J. P. McKelvy, Pittsburgh.*)

(Received for publication, January 24, 1916.)

Since the report by Faust and Tallquist[1] of their studies on the hemolytic action of the unsaturated fatty acids and their soaps, attention has been drawn to the possibility of these substances exercising an important rôle in the grave anemias.

King[2] and Medak,[3] in collaboration with Eppinger, have recently published analyses of the blood lipoids in different diseases and found in the severe anemias an exceptionally high iodine number, which they considered to be characteristic, and which suggested that the hemolysis was due to the unsaturated fatty acids. But their method and calculations are such as to make all their results questionable. I have found the analytical method followed by King in Medak's publication to be, in detail, as follows:

"Es wurden 100 ccm durch Venae punctio entnommenen Blutes defibriniert und mit 1000 ccm 96%igem Alkohol versetzt. Auf diese Weise wurden die Eiweisskörper quantitativ gefällt. Ein Teil der Blutfette ging hierbei in den Alkohol über. Der gesamte Niederschlag wurde vom Alkohol abfiltriert und getrocknet, das Filtrat eingedampft. Der auf dem Filter verbliebene, getrocknete Niederschlag und der Abdampfrückstand wurden im Soxhletapparate mit Petroläther (Kahlbaum) durch 72 Stunden extrahiert. Der Petroläther wurde abgedampft, der auf Gewichtskonstanz gebrachte Rückstand ergab die Menge des in 100 ccm Blut enthaltenen Gesamtfettes. Der Rückstand wurde nun mit heissem 96%igen Alkohol

[1] Faust, E. S., and Tallquist, T. W., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 367.

[2] King, J. H., *Arch. Int. Med.*, 1914, xiv, 145.

[3] Medak, E., *Biochem. Z.*, 1914, lix, 419.

extrahiert, der Extrakt auf ungefähr 50 ccm eingeengt und mit heisser Digitoninlösung (Digitonini cryst.—Merck—1.0: Alkohol 96% 100.0) das Cholesterin ausgefällt, der Niederschlag auf einen vorher gewogenen, mit Asbestwolle beschickten Goochtiegel gebracht, getrocknet und gewogen. Die erhaltene Zahl, durch 4 dividiert, ergab die Menge freien Cholesterins. Nun wurde das beim Abfiltrieren des Digitonincholesterids erhaltene alkoholische Filtrat mit dem auf dem Goochtiegel verbliebenen Niederschlag vereinigt und mit Petroläther extrahiert . . . . ”

Why extract with petroleum ether, after weighing? Or, if the petroleum ether extraction is necessary, and I believe it is, to get the digitonin cholesterids free from lipoid substances, why not weigh after extraction? The first value is certainly incorrect.

“ . . . . der Extrakt eingedampft, der Rückstand in 96%igem Alkohol gelöst und mit Natriumäthylat verseift. Auf diese Weise wurde das an Ester gebundene Cholesterin frei. Nach der Verseifung wurde filtriert [?], das Filtrat [A] abermals mit Petroläther mehrmals extrahiert, der Extrakt eingedampft, in heissem 96%igen Alkohol gelöst und mit heisser 1 %iger alkoholischer Digitoninlösung gefällt, der Niederschlag auf einen vorher gewogenen Goochtiegel gebracht [B], getrocknet und gewogen. Die erhaltene Zahl, durch 4 dividiert, ergab die Menge an Ester gebundenen Cholesterins. Das Filtrat wurde zur Bestimmung der Hüblschen Jodzahl verwendet.”

It is not made clear, but it is likely, that the filtrate designated A and not B was used for the iodine number determination. It is wrong to disregard B entirely because a small amount from the soap of the unsaturated fatty acids goes into the petroleum ether as an impurity. The bulk of the fatty acids existing as soap is in filtrate A. Before extraction, it has to be liberated by acidification, as was done by Medak, but King extracted these soaps after evaporation to dryness, by chloroform.

It is, of course, possible that the hemolysis in anemia is due to unsaturated fatty acids, but as the residue, in which King determined the iodine number, contained fatty acids only as impurities, there seems little basis for the hypothesis that the high iodine numbers were due to unsaturated fatty acids. But one may say that the unknown unsaponified material, which was dissolved by chloroform extraction, contains the substance, which absorbed the iodine and gave the high iodine number. This would be true if the calculation had been correct. To demonstrate this error, I have to again cite from the original article:

"King bestimmte die Jodzahl in folgender Weise: Das Filtrat, das beim Abfiltrieren von dem an Ester gebundenen Cholesterin erhalten wurde, wurde eingedampft, in heissem Chloroform gelöst und auf 100 ccm aufgefüllt. 10 ccm davon wurden mit 25 ccm der Hüblschen Jodlösung versetzt . . . . Die Jodzahl erhielt King aus einem Bruche, in dessen Zähler die Zahl der verbrauchten Kubikzentimeter Natriumthiosulfatlösung, multipliziert mit dem Titer der letzteren, in dessen Nenner eine Zahl stand, die sich aus der Differenz zwischen Gesamtfett und freiem Cholesterin + an Ester Gebundenem Cholesterin ergab. Stets wurden Cholesterin, Cholesterinester und Jodzahl auf 1000 ccm Blut berechnet."

When we consider that the iodine number represents the amount of iodine in gm. absorbed by 100 gm. of fatty material, it is apparent that the calculations of King and Medak are incorrect. King cites in his paper the iodine value of oleic acid as 90, the true iodine number of oleic acid by the usual method of calculation. Yet we find in Medak's quotation[3] of King's work, the iodine number calculated from the number of cc. of 0.1 N $Na_2S_2O_3$ used, divided by the amount of fatty substance in gm., on the basis of 1,000 cc. of blood. King does not mention this calculation in his paper, but only gives the result, leaving the nature of the unsaturated material in doubt.

It is not possible to compare results obtained by two so widely different methods of calculation, so that their high iodine numbers (King 326 and Medak 629) are meaningless, in terms of the usual method of calculation.

Further, I could not find any explanation why King and Medak took only 10 cc. of the chloroform solution, when the reference King mentions[4] advises taking 0.15 to 0.18 gm. of the substance containing unsaturated fatty materials for the iodine number determination. The entire 100 cc. of chloroform solution could not contain more substance than the required quantity, and taking as little as in 10 cc. increases the possibility of error.

To avoid criticism of my results by the use of a different method it was my intention to follow the procedure of King and Medak as nearly as possible in obtaining the extract of the blood fat. Regardless of how the blood is prepared for extraction, or what solvents are used, the extraction is always incomplete by the present methods. Even with the most effective solvent ethyl alcohol, followed by ether or petroleum ether, a small amount is

[4] "Abderhalden's Handb. biochem. Arbeitsmethoden, II."

always found in the residue from the fat and cholesterol. As the isolation of the fat and cholesterol by the saponification method is quantitatively correct, it would eliminate the small error and, if enough material is available, would seem preferable.

Methods.

The blood was taken from the vein 2 hours after a breakfast of tea and toast (without milk or butter), except in Case 2, which was taken 4 hours after a full meal. A few crystals of potassium oxalate were added to prevent clotting. 100 cc. of blood were diluted to 1,000 cc. with 95 per cent alcohol and, after standing 24 hours, were filtered directly into a large extraction shell and extracted by absolute alcohol for 24 hours and then by petroleum ether for 48 hours in a Soxhlet apparatus. The filtrate from the blood protein precipitate, as well as the absolute alcohol and petroleum ether extracts, was evaporated on the water bath to dryness, dissolved again with petroleum ether, and filtered. In the three anemia cases, this petroleum ether extract was evaporated and dried *in vacuo* over sulfuric acid to constant weight (in Table I, fat + lipoids). In the hemolytic jaundice, the petroleum ether extract was diluted to 100 cc. and only 50 cc. were used for determining the fat + lipoids. Windaus'[5] digitonin method, as modified by Fraser and Gardner,[6] was used for the cholesterol determination.

For the saponification of the esters of cholesterol, the method as described by Gephart and Csonka[7] for fatty acids was used, naturally without acidifying before extraction by ether (A). The ether extract was washed with distilled water until neutral (B), the ether extraction residue dissolved in alcohol, and the cholesterol (combined) determined as before. The alkaline solution A, containing the chief bulk of fatty acids, was acidified and extracted with ether (first with the ether used for washing the cholesterol digitonin—as combined—precipitate) and then combined with the ether extract of the wash water B and the ether extract of the

[5] Windaus, A., *Z. physiol. Chem.*, 1910, lxv, 110.

[6] Fraser, M. T., and Gardner, J. A., *Proc. Roy. Soc., Series B*, 1909–10, lxxxii, 560.

[7] Gephart, F. C., and Csonka, F. A., *J. Biol. Chem.*, 1914, xix, 521.

wash water from the digitonin cholesterids (as combined); the two latter were also first acidified to liberate the fatty acids. The ether extract, after washing free from hydrochloric acid, was evaporated and dried *in vacuo* over concentrated sulfuric acid to constant weight (Table I, fatty acids). This dissolved in 10.0 cc. chloroform served for the iodine number determination according to Hübl.

In Case 4, as mentioned before, 50 cc. of the petroleum ether extract were used for the direct determination of the iodine number, as it was thought that the highly unsaturated fatty acids might have been destroyed by the saponification. It was found

TABLE I.

In 1,000 Cc. of Blood.

Case.	Fat + lipoids.	Cholesterol.			Fatty acids.	Iodine number on 100 gm. fatty acids.	Remarks.
		Free.	Combined.	Total.			
	gm.	*gm.*	*gm.*	*gm.*	*gm.*		
1	4.7860	0.2110	0.5820	0.7930	2.284	53.36	Pernicious anemia.
2	6.9730	0.6448	0.8144	1.4592	4.934	56.43	" "
3	5.6575	0.5759	0.7129	1.2888	2.937	70.43	" "
4	6.9100	0.7196	0.5144	1.2340	2.500	103.30	Congenital hemolytic jaundice.

that 0.3455 gm. extract absorbed 0.2618 gm. iodine, giving an iodine number of 75.76; but 0.0405 gm. iodine was used by 0.0617 gm. cholesterol present. Therefore, the iodine number of the extract minus the cholesterol $\left(\frac{0.2213 \times 100}{0.2838}\right)$ is 98.17, corresponding to the iodine number of the fatty acids of 103.3 (see Table I) obtained after saponification. Thus, either by the direct determination or after saponification we get in congenital hemolytic jaundice an iodine number only a little higher than that of oleic acid. To determine the nature of the substance which is responsible for the absorption of iodine a further isolation is necessary. This project will be reported later.

As pointed out in the introduction, the extraction of the blood by any combination of the best solvents is never complete. To demonstrate this, 10 cc. of the whole blood were saponified, as described by Gephart and Csonka,[7] for the fatty acids, and

another 10 cc. were used for the total cholesterol determination, by extraction from the alkaline solution. On the other hand, two samples were taken of the dried blood protein residue after extraction (obtained from 100 cc. blood) and saponified, one for cholesterol and the other for the fatty acids determination. The results are given in Table II.

The form in which the cholesterol was present, free or combined, could not be determined, but it represents, in the extraction method of cholesterol determination as in that of the fatty acids, an error of over 10 per cent.

Though the results will be published later when more cases of other pathological conditions are collected, I wish to describe a modification of the Grigaut[8] and Autenrieth-Funk[9] colorimetric

TABLE II.

In 1,000 Cc. of Blood (Case 4).

	Extract.	Residue.	Total.	Saponification.	Colorimetric.
	gm.	*gm.*	*gm.*	*gm.*	*gm.*
Fatty acids	2.500	0.3276	2.8276	2.7646*	
Cholesterol (total)	1.2340	0.1432	1.3772	1.4330	1.415

*0.1 N cc. $\times$ 0.0274.

methods of estimation of blood cholesterol, a method used successfully in this laboratory during the past year.

2 cc. of blood measured by an Ostwald pipette are placed in a flask (Fig. 1). 20 cc. of alcohol (95 per cent), 4 gm. of potassium hydroxide, and 2 cc. of a 10 per cent solution of barium chloride are added. The contents are boiled under a reflux condenser for 1 hour, cooled, distilled water is added to the constriction (about 50 cc.), and 50 cc. of ether are added. The flask is closed with the stopper and shaken in a rotary manner for a few minutes. After the ether layer has separated it is blown by pressure into a separating funnel (250 cc.). The ether extraction is repeated twice with 50 cc. portions, collecting the portions in the separating funnel. After each extraction the ether layer is brought to the constriction by adding a little distilled water. The com-

[8] Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxxi, 513.

[9] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

bined ether extract in the separating funnel is washed with several portions of water until the wash water is neutral. The turbid ether extract after being filtered into an Erlenmeyer flask is perfectly clear and colorless. After the ether is distilled off the flask is placed in a water bath for half an hour. When the flask is cooled off, the residue is dissolved in 3 cc. portions of chloroform (at room temperature), each portion is filtered through a 5.5 cm. diameter filter paper, and collected in a 10 cc. normal flask. The flask is filled up to the mark with chloroform and 5 cc. are placed in another 10 cc. normal flask to serve as a control.

FIG. 1. a. Upper bulb, about 130 cc.
b. Lower bulb, 70 cc.
c. Constriction, 25 mm. diameter.

A cholesterol solution containing 1 mg. of pure cholesterol in 5 cc. of chloroform is used as a standard. 2 cc. of acetic anhydride and 0.1 cc. concentrated H_2SO_4 are added to each of the flasks, which are placed in a dish of water (30°C.), and let stand in the dark for 15 minutes. After this the flasks are cooled rapidly, filled with chloroform to the 10 cc. mark, and the color is compared immediately by the Duboscq colorimeter, setting the standard at 15 mm. It is necessary to use tubes with screw attachment.

The above described procedure of adding $BaCl_2$ to the saponi-

fication gives a very satisfactory reading as it eliminates almost entirely the disturbing yellowish color.

SUMMARY.

In three cases of pernicious anemia the fatty acids of blood gave an average iodine number of 60. In one case of congenital hemolytic jaundice the iodine number 103.3 was found.

The high iodine number in pernicious anemia and congenital hemolytic jaundice reported by King and Medak is based on incorrect calculation.

The blood cholesterol in all four cases is considerably lower than that of a normal individual, which agrees with the findings of previous authors.

A modification of the colorimetric determination is presented.

DETERMINATION OF CREATINE IN MUSCLE. II.

By LOUIS BAUMANN and HARRY M. HINES.

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(Received for publication, February 5, 1916.)

From the results of their experimental work, Janney and Blatherwick[1] conclude that "the principle of hydrolysis with acids is to be avoided in the estimation of creatine in complex tissues." They recommend an extraction method which they claim to be accurate and simple. A comparison of the figures obtained by this method with those obtained by an acid hydrolysis method, reported by one of us two years ago[2] fails to show any marked difference between the two. In the case of dog muscle, which was chiefly used by Janney and Blatherwick for their determinations, the agreement is striking. The average for dog muscle (sixteen different animals) corresponds with that reported by Myers and Fine.[3]

The older method has now been simplified so that a complete determination may be carried out in less than ½ hour after the extract is obtained. This method may be used with advantage for the estimation of the sum of creatine and creatinine in organs. The results when expressed in terms of 100 gm. of moist organ are a few mg. higher than those obtained by the Janney and Blatherwick method.

The apparent objections to the Janney and Blatherwick method are that it is occasionally difficult to obtain a representative 5 gm. sample and also that filtration of certain organ extracts according to this method is extremely slow.

It is a matter of considerable importance to know if the Jaffé reaction as it is obtained with the hydrolyzed muscle extract is

[1] Janney, N. W., and Blatherwick, N. R., *J. Biol. Chem.*, 1915, xxi, 567.
[2] Baumann, L., *J. Biol. Chem.*, 1914, xvii, 15.
[3] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1913, xiv, 9.

to be attributed to creatinine alone. We attempted to precipitate this substance as the potassium picrate salt. We find that all the substance in the hydrolyzed muscle extract which gives the Jaffé reaction may be precipitated in this way.[4] Janney and Blatherwick suggest that acetyl-propionic acid which also gives the Jaffé reaction, may be formed when muscle tissue is heated with solutions of mineral acids. When 50 gm. of muscle together with 0.5 gm. of glucose are heated for 3 hours with 5 N sulfuric acid according to Baumann, no increase in the colorimetric reading is observed.[5]

EXPERIMENTAL.

Comparison of the Two Methods.

The creatine content is expressed in mg. per 100 gm. of moist muscle or organ.

Dog muscle.		Beef muscle.		
Janney and Blatherwick.	Baumann.	Janney and Blatherwick.	Baumann.	
mg.	*mg.*	*mg.*	*mg.*	
376	381	391	412	
362	379	462	451	
386	381	490	488	
380	383	415	421	
376	378	435	425	
366	365	427	433	
386	384	455	450	
389	390	373	369	(much connective tissue.)
376	370	407	411	
365	362	439	450	
348	353	Average 429.4	431.0	
353	353			
316	328			
379	371			
381	394			
367	365			
Average 369.1	371.0			

[4] This work was carried out by Mr. Ingvaldsen. The quantitative data will appear in a subsequent article.

[5] 0.5 gm. of glucose is more than the sum of glucose plus glycogen in 50 gm. of muscle.

Cat muscle.		Rabbit muscle.	
Janney and Blatherwick.	Baumann.	Janney and Blatherwick.	Baumann.
mg.	*mg.*	*mg.*	*mg.*
472	477	500	485
492	497	537	543
497	504	547	548
Average 487	492.7	531	534
		Average 528.7	527.5

	Janney and Blatherwick.	Baumann.
	mg.	*mg.*
Dog brain....	108	120
Beef "	103	113
" testicles.	175	195
Sheep " .	203	215
" " .	216	209
Dog liver.....		28.2
" "		24.3
" "		23.6
" "		21.0

The Effect of Added Glucose to Muscle before Hydrolysis.

50 gm. of hashed beef muscle to which 0.5 gm. of glucose had been added were boiled for 3 hours under a reflux with 125 cc. of 5 N sulfuric acid and the creatinine was determined as described below. The control contained 433 mg. of creatine per 100 gm. of mu-cle, whereas the muscle plus sugar contained 431 mg.

The Simplified Method for the Determination of the Sum of Creatine and Creatinine in Muscle.

The technique is similar to that already described,[2] except that 10 cc. of the extract are used instead of 20 cc. This makes evaporation unnecessary. The procedure is briefly as follows: 10 cc. of the hydrolyzed and filtered extract are pipetted into a 50 cc. volumetric flask, 9 cc. of 10 per cent sodium hydroxide solution added, the solution is cooled, and then diluted to the mark with saturated picric acid solution. The contents of the flask are well mixed, then filtered. The creatinine is determined according to Folin. 25 cc. of the clear filtrate and the requisite amount of standard solution are pipetted respectively into 250 cc. volumetric

flasks. 15 cc. of saturated picric acid solution and sufficient distilled water to equalize the volumes in both flasks are added to the standard. The color is developed by adding 2.5 cc. of 10 per cent sodium hydroxide solution to the standard and 3 cc. to the unknown. After 10 minutes the solutions are diluted to the mark and the color intensities compared. The standard is set at 10 mm. The standard consists of a 0.1 N hydrochloric acid solution containing the equivalent of 1 mg. of creatinine per 1 cc. of solution. We have employed the creatinine zinc chloride salt for this purpose. For the determination of dog muscle 3 cc., for beef 3.5 cc., and for rabbit muscle 4.5 cc. of this solution are required.

The results obtained by this procedure are practically identical with those obtained by the older method.[2]

	Older method.	Newer method.
	mg.	*mg.*
	385	382
	388	383
	457	467
	384	389
	341	343
Average.....	391	393

SOME INDICATORS FROM ANIMAL TISSUES.

By W. J. CROZIER.

Contributions from the Bermuda Biological Station for Research, No. 44.

(Received for publication, February 2, 1916.)

Indicators occurring in nature have for the most part been found in plants; in fact such materials have seldom been recorded from animal sources. An examination of a number of pigmented animals, however, shows that this rarity is by no means so great as has been supposed. The following notes refer to prep-

TABLE I.

Color Changes of Some Indicators from Animal Tissues.

Source.	Acidity.*								
	p_H=5.0	5.3	5.6	5.9	7.4	7.6	7.65	8.0	10.0
Ascidia atra.....	Red.	(Increasingly red.) ←			Red.	Green (ppt.).			
Ptychodera sp.....							Yellow.	Brown.	
Chromodoris zebra			Pink.	Blue.	Blue.	Blue.	Blue (green ppt.).		
(?) *Eupolymnia aurantiaca*.....								Red.	Green.

* Observations were made at a number of intermediate points.

arations obtained from some marine animals, which give clear-cut[1] reversible color changes as summarized in Table I. The hydrogen-ion concentrations observed were obtained by acetate, borate, and phosphate mixtures, at 20–25°, with the usual indicators for comparison (Michaelis, 1914). The tests were made by

[1] A number of other soluble pigments have been found in which an acid-alkali color change is undoubtedly present, but is not sufficiently clear-cut to be of use. For example, a sponge, *Tedania ignis*, gives brown extracts, which are made distinctly red by acids.

adding two drops of a concentrated solution of pigment to 10 cc. of the different mixtures.[2]

1. Ascidia atra.—This common black tunicate of Bermuda and the West Indies contains in its test a substance readily extracted by acetone, less easily by alcohol or glycerol, but not dissolved by ether or chloroform. This pigment is for the most part closely packed in the outer tenth of the thickness of the test, and occurs in the form of rather large granules carried by cells in the test canals. Its color is a deep purple, as may be seen when prepared sections of the test are studied microscopically. The pigment is insoluble in sea water, but observations indicate that it is excreted in granular form from the surface of the animal. According to Dahlgrün (1901), in many tunicates an excretory function is maintained by wandering mesenchymal cells the protoplasts of which bear pigment granules.

Solutions of the pigment are dark red, this color being intensified by acids and turned green by alkalies, which also precipitate the substance in greenish flocculent masses.

There is probably some relation between this dark purplish red substance and the pigment, described by Herdman (1913) and Holt (1914), which occurs in the test of the compound ascidian *Diazona violacea*,[3] although the *Ascidia* pigment does not appear to show any absorption bands.

2. Ptychodera sp.—This yellowish balanoglossid when strongly stimulated secretes a yellowish material which turns reddish brown at about $p_H = 8.0$. Extracts of the animal, containing this pigment, can be made with alcohol, distilled water, or aqueous solvents, but they are not useful as indicators because the brown color is permanently assumed on standing for some time. But

[2] Useful indicators have also been prepared from several Bermuda plants, as the berries of the sage bush (*Lantana involucrata*), which yield on extraction with alcohol a bright red solution, changing to yellow (alkaline) at $p_H = 6.6$; the fruit of the cactus (*Opuntia*) also contains a red material changing to lemon yellow (alkaline) at $p_H = 9.0$; the juice from the ink berry of the sand beaches (*Randia aculeata*) is a splendid indicator, being dark red with acids and changing to a brilliant green (alkaline) at $p_H = 8.5$.

[3] Coloring matter precisely like that of *Diazona violacea* occurs in Bermuda ascidians which are closely related to this species.

within the tissues of the living animal the change resulting from the penetration of alkalies is well shown.

3. Chromodoris zebra.—The indicator contained in this nudibranch has been described elsewhere (Crozier, 1914, 1915, 1916), but for comparison its color changes have been included in Table I.

4. Eupolymnia aurantiaca (?).—An orange red annelid, probably *Eupolymnia aurantiaca,* which is occasionally found among blocks of coral rock in which it burrows, gives with aqueous alcohol a bright red extract of the integument, which changes to orange on standing for some days. It shows a sharp transition from red to bright green at about $p_H = 10.0$, though even at higher acidities a precipitate (which consists of the pigment substance) appears slowly; but the color change in the solution appears only above $p_H = 10.0$. The red solutions transmit only a narrow portion of the red and of the yellow, while the green acid solutions transmit only the green-blue.

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FAT ASSIMILATION.

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(Received for publication, February 23, 1916.)

Our conception of the changes which ingested fat undergoes in the animal body is fairly clear as regards the beginning and the end but very vague as to the intermediate stages. The way in which the food fat (or at least the greater part of it) gets from the intestine into the blood has been quite satisfactorily determined. It is saponified in the intestine, absorbed in water-soluble form as soaps and glycerol, resynthesized by the intestinal cells, and passed into the chyle and thence to the blood as neutral fat (glycerides) suspended in the plasma in a very finely divided condition. About 60 per cent of the food fat has been actually accounted for in the chyle in this way and this figure is probably low. The remaining smaller quantity is generally assumed to be absorbed directly into the blood stream by way of the intestinal capillaries. Support is given to this assumption by the work of Munk and Friedenthal[1] who found that the blood fat increased after the thoracic duct had been ligated, also by the finding of d'Errico[2] that the fat content of the portal vein is always higher than that of the jugular during fat absorption, and by the work of Hamburger[3] who found that the absorption of soaps from an isolated loop of intestine took place after all visible lymph vessels had been tied off.

There is also a quite satisfactory hypothesis regarding the later stages of fat metabolism based on the work of Knoop[4] and of

[1] Munk, I., and Friedenthal, H., *Zentr. Physiol.*, 1901, xv, 297.

[2] d'Errico, G., *Arch. fisiol.*, 1907, iv, 513.

[3] Hamburger, H. J., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1900, 554.

[4] Knoop, F., *Beitr. chem. Phys. u. Path.*, 1905, vi, 150.

Leathes and his coworkers.[5] The long chain fatty acids are desaturated in the liver and are then broken down to carbon dioxide and water by successive oxidations. Whether the long chains are broken into shorter ones at the points of desaturation previous to oxidation is a disputed point. At some stage in the oxidation carbohydrates appear necessary for complete combustion.

But as to what happens to the fats from the time they enter the blood stream until they reach the desaturation stage we have very little information of any kind. The suspended fat persists in the blood for some hours and then disappears. Some part of it goes to the liver, whose fat content increases during fat absorption. The blood serum 10 to 12 hours after a meal is clear and free of suspended particles, and remains so during fasting, although considerable quantities of fat must be passing from the fat stores into the blood for the use of the organism, especially after the 2nd or 3rd day of the fast, when the energy requirement is supplied mainly by the stored fat. Because of the fact that the serum remains clear during the transference it is probable that the fat is not being carried as such but as some derivative more readily miscible with water, possibly lecithin.

Ever since the chemical similarity between lecithin and the fats was discovered, attempts have been made to connect the two in metabolism; but although there has been much speculation no definite evidence has been offered. In fact lecithin has been generally classed as a cyto-lipoid, having to do more with the structure of the cell than with its processes. However, the idea that there is a dynamic relation between lecithin and fat in normal metabolism has been a persistent one and has continued to stimulate investigation so that recently evidence has been brought forward[6] definitely connecting the two. The evidence consisted of fat absorption experiments in which it was found that lecithin increased in the blood during the absorption of the fat. It seemed probable at the time, in view of the work of Leathes and others, that the seat of lecithin formation was the liver, in which case the

[5] Leathes, J. B., and Meyer-Wedell, L., *J. Physiol.*, 1909, xxxviii, p. xxxviii. Hartley, P., *J. Physiol.*, 1909, xxxviii, 353.

[6] Bloor, W. R., *J. Biol. Chem.*, 1915, xxiii, 317.

observed increases in lecithin would probably be more marked in the plasma than in the whole blood, since the plasma is ordinarily regarded as the carrier.

The present work is a continuation of the above, carried out in order to get further information regarding the process and to locate if possible the seat of formation of the lecithin.

Dogs were given a feeding of fat only, with the exception of Experiment 4 where water was given. Blood samples (10 cc.) were taken at once and at 2 hour intervals for 8 hours. Comparative analyses of whole blood and plasma for total fat, cholesterol, and lecithin were made as follows.

The sample was drawn from the jugular vein into a 10 cc. pipette (containing a little powdered potassium oxalate) connected with the needle by means of a short length of rubber tubing, and run at once into a 15 cc. centrifuge tube also containing a little oxalate. After mixing, 3 cc. of the blood were measured with a pipette and run slowly (a slow stream of drops) into 75 cc. of a mixture of three parts alcohol and one part ether (both redistilled) in a 100 cc. graduated flask which was kept in motion during the process. The contents of the flask were raised to boiling by immersion in a boiling water bath (with constant shaking to avoid superheating), cooled to room temperature, made to volume with alcohol-ether, mixed, and filtered.

The remainder of the blood was then centrifuged for a definite time, the relative volume of corpuscles and plasma noted, and 3 cc. of the plasma were then measured out and treated in exactly the same way as the whole blood.

The alcohol-ether extracts of whole blood and plasma so obtained were sufficient for duplicate determinations of total fat, cholesterol, and lecithin which were made as follows.

Total Fat.—(Total fatty acids plus cholesterol.) For this determination 10 cc. of the extract were used. The procedure was the same as has been reported several times recently[7] and therefore need not be repeated here. The value "total fatty acids" used in the table was obtained by subtracting the value obtained for cholesterol from that of total fat.

[7] Bloor, *J. Biol. Chem.*, 1915, xxiii, 317, and earlier papers.

Cholesterol.—The method used for the determination of cholesterol has been reported recently.[8] It consists of an application of the Liebermann-Burchard color reaction as developed by Autenrieth and Funk[9] to the alcohol-ether extract of the blood and plasma. 10 cc. of the blood extract were used for the determination.

Lecithin.—The method used has been reported previously[10] but since in some of the present work a variation of the method (use of strychnine molybdate precipitation for the phosphoric acid) has been employed it is desirable to give the procedure in detail. It is as follows: 10 cc. of the extract of whole blood or 15 cc. of extract of plasma are measured with a pipette into a 200 × 25 mm. Jena test-tube, two or three glass beads of about 3 mm. diameter are added, and the solution is evaporated to dryness by immersion of the tube in a water bath. The tube should be shaken frequently until boiling has begun, after which the evaporation will proceed to dryness without further attention. The residue should be dried in the tube for 15 minutes to remove traces of alcohol which might interfere with the completeness of the subsequent oxidation. For the oxidation there are now added to the tube 1.5 cc. of a mixture of equal parts of concentrated sulfuric and nitric acids and the whole is digested by heating with a micro burner in the hood or with a Folin draught apparatus.[11] The heating is done in two stages. During the first the mixture is gently boiled with a very low flame until the red fumes have ceased to come off. This should take about 15 minutes (instead of 5 minutes as given in the original description). If the process is hastened too much at this stage the nitric acid will be driven off before oxidation is complete. The tubes should be inclined at an angle of about 30° to prevent loss by spattering. The heat is then gradually raised until the nitric acid is completely driven off, after which the solution is boiled for 8 to 10 minutes. The mixture is then cooled somewhat and two drops of 0.25 per cent cane-sugar solution are added (to destroy a nitric-phosphoric acid combination), after which the heating is resumed for

[8] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

[9] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

[10] Bloor, *J. Biol. Chem.*, 1915, xxii, 133.

[11] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 503.

about 1 minute. The sugar should cause a slight browning of the liquid which quickly disappears on heating. The tube is then cooled and about 3 cc. of water are added, washing down the sides of the tube.

The phosphoric acid is determined nephelometrically either by precipitation as the silver salt as in the original method or as strychnine phosphomolybdate, using Kober and Egerer's[12] modification of the Pouget-Chouchak reagent. The results by the two precipitations are practically identical. The silver precipitation was used in the first six experiments, the strychnine molybdate in the remaining one. The procedure is somewhat different for the two precipitations and is as follows.

Silver Precipitation.—To the solution in the Jena test-tube is added a drop of phenolphthalein (0.3 per cent) and the acid is exactly neutralized as follows. 20 per cent NaOH (free from chlorides and containing but little carbonate) is added to neutralization, noting the amount used, 0.5 N sulfuric acid run in till just acid, the solution cooled, and 0.1 N NaOH added until it is just alkaline again. To the neutralized solution are added 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. of 0.1 N NaOH and the solution is made to 10 cc. (indicated with sufficient accuracy by a scratch on the tube). A standard phosphate solution is similarly prepared as follows. 3 cc. of a phosphate solution (containing 0.15 mg. H_3PO_4) are measured into a similar Jena test-tube, a drop of phenolphthalein is added, and then the amount of 20 per cent alkali that was used for neutralization of the test solution is run in. Concentrated sulfuric acid is added till just acid, the excess of acid neutralized by a drop or two of the strong alkali, the solution cooled, and then exactly neutralized as above. 1 cc. of 10 per cent $(NH_4)_2SO_4$ and 1.5 cc. of 0.1 N NaOH are added and the solution is made to the 10 cc. mark on the tube. Two samples of 10 cc. each of 2 per cent $AgNO_3$ (neutral) are measured into 25 cc. glass stoppered graduated flasks and the standard and test solutions in the Jena test-tubes are added through a funnel with the stem drawn out so that the 10 cc. are delivered in about 15 seconds. The liquid in the flask is gently rotated while the phosphate solution is being run in, then the test-tube

[12] Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2373.

rinsed out with water, and the wash water poured in through the funnel. Finally the liquid in the flask is brought up to the mark by rinsing the funnel with distilled water and the whole well mixed, when it is ready to read in the nephelometer.

Strychnine Molybdate Precipitation.—For this precipitation Kober's[12] strychnine molybdate reagent in HCl was used. Contrary to Kober's findings good results had been obtained nephelometrically with the reagent as originally devised by Pouget and Chouchak[13] but Kober's modification is so much of an improvement that there is no longer any reason for using the original. As was pointed out[14] for the original reagent, account must be taken of the salt content, reaction, etc., of the solutions because of the effect of these factors on the nature of the precipitate, and for that reason certain modifications in Kober's procedure are necessary. The process as applied in this work is as follows.

To the solution in the Jena test-tube is added a drop of phenolphthalein and it is then neutralized with 20 per cent NaOH as before, noting the amount added. The solution is then made just acid with a drop or two of 1 : 1 HCl, cooled, transferred quantitatively to a 25 cc. graduated flask, and made up to volume.

3 cc. of the standard phosphate solution are measured into another 25 cc. graduated flask, a drop of phenolphthalein is added, and the amount of 20 per cent NaOH used in the test solution run in. The solution is then neutralized with concentrated H_2SO_4, cooled, and made to volume. 10 cc. of each solution are used for the precipitation, which is carried out according to Kober's directions except that as a consequence of the salt content of the solution a stronger acid must be used to prevent decomposition of the reagent.

The precipitation is carried out as follows: To each of two 50 cc. graduated flasks are added about 25 cc. of distilled water, 5 cc. of 1:1 HCl, and 5 cc. of the molybdate reagent; then 10 cc. of the phosphate solutions are measured in with a pipette, keeping the flasks gently rotating during the addition. Water is added to the mark, the solutions are mixed by cautiously inverting several times, and, after standing 3 minutes, compared in the nephelometer.

The strychnine molybdate reagent possesses the advantage that while more sensitive to phosphoric acid than the silver reagent it is less sensitive to impurities. The values obtained, multiplied by eight, give a close approximation to the phosphatide values.

[13] Pouget, I., and Chouchak, D., *Bull. Soc. chim.*, series 4, 1909, v, 104.
[14] Bloor, *J. Biol. Chem.*, 1915, xxii, 135.

Direct determinations were made on both whole blood and plasma from which, knowing the percentage of corpuscles in the blood the composition of the corpuscles was calculated. Direct determinations on the corpuscles by the above procedure do not appear feasible because of the apparently unavoidable clumping[15] of the corpuscles during the extraction. The error in each of the above methods as ordinarily carried out is 3 to 5 per cent.

Experiments were carried out on two different dogs as follows.

Experiment 1.—Dog 25, a female bull terrier, weight 8.35 kg., in very poor condition, was fed 50 cc. of olive oil (forced feeding)[16] at 8.55 a.m. The 1st sample of blood was taken at 9.00 a.m. After taking a 3 cc. sample for whole blood the remainder was centrifuged for 7 minutes at 2,000 R. P. M. The corpuscles were found to be 35 per cent. The plasma was slightly cloudy and yellow in color. A 3 cc. sample was taken for analysis.

		Corpuscles. *per cent*	Plasma.
2	11.00 a.m.	37	Yellowish white and cloudy.
3	1.00 p.m.	37	Milky white.
4	3.00 "	35	Less milky than in 3.
5	5.00 "	37	More milky.

Experiment 2.—Dog 25 as before. Fed 60 cc. of olive oil at 8.45 a.m.

1st blood sample at 8.50 a.m., centrifuged 10 minutes at 2,000 R. P. M. Corpuscles 34 per cent. Plasma cloudy. Other samples as follows:

		Corpuscles. *per cent*	Plasma.
2	10.50 a.m.	35	Cloudy.
3	12.50 p.m.	35	Milky.
4	2.50 "	34	"
5	4.50 "	36	Slightly cloudy.

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[15] Later experiments have shown that the clumping may probably be avoided by more careful addition of the corpuscles to the alcohol-ether.

[16] A method of forced feeding of liquids which has been found very useful, especially for dogs, was suggested by Mr. G. L. Foster of this laboratory. The dog's head is held with one hand while with the other the lips are pulled away from the jaws on one side producing a pouch into which the liquid is poured and from which it runs into the throat and is swallowed naturally. This method does away with the necessity of a stomach tube and avoids the complications which occasionally accompany its use, especially by beginners.

Experiment 3.—Dog 25, weight 8.88 kg. (gain of 0.52 kg. since last experiment). Fed 55 cc. of olive oil at 8.45 a.m.

1st sample at 8.50 a.m., centrifuged 10 minutes at 3,800 R. P. M. Corpuscles 36 per cent. Plasma clear.

		Corpuscles. *per cent*	Plasma.
2	10.45 a.m.	32	White, cloudy.
3	12.45 p.m.	30	Milky.
4	2.45 "	30	White, cloudy.
5	4.45 "	30	Milky.

About 10 cc. of the oil were lost by vomiting during the first period. This centrifuge at the above speed and time was used for the remainder of the experiments.

Experiment 4.—Dog 25 as above. Fed 55 cc. of olive oil at 8.45 a.m.

1st sample at 9.00 a.m. Corpuscles 32.5 per cent. Plasma clear. About 200 cc. of water were given at this time. Other samples as follows:

		Corpuscles. *per cent*	Plasma.
2	10.50 a.m.	33	White, cloudy.
3	1.00 p.m.	32.5	Milky.
4	2.45 "	Not determined.	Nearly clear.
5	4.45 "	30	Milky.

Experiment 5.—Dog 26, female collie in good condition, weight 17 kg. Fed 100 cc. of olive oil at 8.45 a.m.

1st blood sample at 8.50 a.m., centrifuged as above. Corpuscles 45 per cent. Plasma clear.

		Corpuscles. *per cent*	Plasma.
2	10.45 a.m.	45	Somewhat milky.
3	12.45 p.m.	44	Milky.
4	2.45 "	40	Slightly milky.
5	4.45 "	40	Milky.

Experiment 6.—Dog 26. Fasted 1 day (40 hours since last feeding). Fed 100 cc. of olive oil at 8.40 a.m.

		Corpuscles. *per cent*	Plasma.
1	8.45 a.m.	50	Clear.
2	10.45 "	44	White, cloudy.
3	12.45 p.m.	44	Milky.
4	2.45 "	44	Slightly white, cloudy.
5	4.40 "	44	Milky.

Experiment 7.—Dog 26. Fed 100 cc. of olive oil at 8.40 a.m.

		Corpuscles. *per cent*	Plasma.
1	8.45 a.m.	48	Clear.
2	10.45 "	44	White, cloudy.
3	12.45 p.m.	44	Milky.
4	2.45 "	44	"
5	4.45 "	44	Nearly clear.

In view of the recognized effect on the blood sugar, of excitement and struggling, and of a possible similar effect on blood fat, the dogs used were previously accustomed to the handling so that during the experiments they did not struggle or show other evidences of excitement. It is believed, therefore, that this factor was practically eliminated. Still an occasional high value in the first sample (as for example, Experiment 7, total fatty acids in whole blood) may have been due to this cause.[17]

Attention is called to the changes in percentage volume of the corpuscles during certain of the experiments, particularly noticeable in Experiments 3, 5, 6, and 7. The change is always a decrease, amounting in some cases to more than 15 per cent. The samples were centrifuged in the same centrifuge and for the same periods of time and at the same speed. Tests of the speed of the centrifuge from time to time on different days and times of the day showed that it did not change notably. Also in the experiments noted above no water was given so that there can be no question of dilution of the blood from that source. These changes in percentage of the corpuscles are in all probability due to increases in plasma volume as a result of the increased flow of lymph into the blood by way of the thoracic duct during fat absorption. The varying figures show that the volume of the blood cannot be assumed to remain constant during experiments of this kind.

The analytical results of the experiments are given in Table I below.

[17] This factor may also have been responsible for the lower and more irregular results obtained in the previous experiments.

TABLE I.

The Blood Lipoids during Fat Absorption.

Gm. per 100 cc.

No. of experiment.	Time.	Total fatty acids.			Lecithin.			Cholesterol.		
		Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.	Whole blood.	Plasma.	Corpuscles.
1	Before..	0.52	0.57	0.43	0.32	0.30	0.36	0.20	0.15	0.31
	2 hrs....	0.63	0.67	0.56	0.38	0.30	0.51	0.20	0.16	0.28
	4 " ...	0.97	0.92	1.05	0.54	0.34	0.88	0.22	0.18	0.31
	6 " ...	0.68	0.58	0.85	0.47	0.32	0.72	0.22	0.19	0.30
	8 " ...	0.71	0.75	0.64	0.40	0.32	0.52	0.22	0.19	0.30
2	Before..	0.63	0.60	0.69	0.35	0.29	0.47	0.23	0.20	0.29
	2 hrs....	0.69	0.60	0.86	0.41	0.30	0.62	0.23	0.20	0.29
	4 " ...	0.72	0.61	0.92	0.48	0.30	0.85	0.22	0.19	0.28
	6 " ...	0.77	0.68	0.94	0.48	0.30	0.83	0.21	0.19	0.28
	8 " ...	0.76	0.64	0.70	0.34	0.30	0.44	0.21	0.21	0.28
3	Before..	0.53	0.54	0.50	0.32	0.28	0.41	0.18	0.16	0.22
	2 hrs....	0.59	0.59	0.59	0.36	0.28	0.53	0.18	0.16	0.22
	4 " ...	0.71	0.69	0.76	0.41	0.30	0.67	0.19	0.16	0.26
	6 " ...	0.58	0.61	0.54	0.42	0.30	0.65	0.19	0.16	0.26
	8 " ...	0.61	0.69	0.46	0.37	0.31	0.49	0.19	0.16	0.26
4	Before..	0.53	0.57	0.45	0.32	0.33	0.31	0.19	0.15	0.28
	2 hrs....	0.57	0.59	0.53	0.34	0.35	0.32	0.17	0.15	0.21
	4 " ...	0.75	0.72	0.81	0.41	0.37	0.50	0.17	0.15	0.21
	6 " ...	0.75	0.72	0.81	0.36	0.30	0.47	0.17	0.16	0.19
	8 " ...	0.70	0.62	0.87	0.40	0.28	0.65	0.18	0.16	0.25
5	Before..	0.63	0.70	0.65	0.32	0.30	0.31	0.30	0.31	0.29
	2 hrs....	0.76	0.79	0.73	0.37	0.34	0.40	0.29	0.31	0.27
	4 " ...	0.93	0.99	0.84	0.41	0.34	0.50	0.28	0.31	0.24
	6 " ...	0.83	0.86	0.81	0.36	0.36	0.36	0.30	0.30	0.30
	8 " ...	0.90	0.93	0.86	0.36	0.33	0.40	0.28	0.31	0.24
6	Before..	0.68	0.76	0.60	0.40	0.50	0.30	0.30	0.37	0.23
	2 hrs....	0.80	0.91	0.66	0.47	0.53	0.40	0.29	0.36	0.20
	4 " ...	1.08	1.15	1.00	0.53	0.51	0.56	0.28	0.34	0.20
	6 " ...	0.89	0.90	0.88	0.46	0.52	0.40	0.29	0.35	0.21
	8 " ...	0.95	0.96	0.94	0.48	0.51	0.44	0.28	0.33	0.22
7	Before..	0.64	0.62	0.66	0.40	0.44	0.36	0.21	0.23	0.19
	2 hrs....	0.62	0.56	0.70	0.41	0.44	0.38	0.23	0.25	0.19
	4 " ...	0.90	0.95	0.84	0.49	0.43	0.56	0.23	0.25	0.19
	6 " ...	0.73	0.86	0.75	0.42	0.39	0.46	0.23	0.24	0.22
	8 " ...	0.81	0.73	0.91	0.42	0.42	0.42	0.23	0.24	0.22

DISCUSSION.

Total Fatty Acids.—(Combined in various forms—glycerides, lecithin, cholesterol esters—and free, as soaps or fatty acids.) The increases in whole blood and plasma are those long known to take place in the blood during fat feeding (alimentary lipemia). The greatest increase noted in the above experiments was 86 per cent for whole blood and 61 per cent for plasma. More striking, however, are the increases in total fatty acids in the corpuscles. With two exceptions (Experiments 5 and 7) these are much greater than in either whole blood or plasma. In Experiment 1 the greatest increase in whole blood is 86 per cent, plasma 61 per cent, corpuscles 144 per cent; in Experiment 4, the increases are, whole blood 42 per cent, plasma 27 per cent, corpuscles 86 per cent. These results bear out the findings of Munk[1] who in 1901 reported that the fat of the corpuscles increased during fat absorption and indicate that the corpuscles actively absorb the fat from the plasma.

Another point shown in most of the above experiments and referable probably to the way in which the liquid fats pass from the stomach is the decrease in the fat of whole blood and plasma at the fourth period (6th hour) after feeding and the increase again at the fifth period. Apparently the larger quantity of oil passes at once to the intestine like other liquids and the remainder not till a later period (when the hunger contractions begin?).

Cholesterol (Total).—The changes in cholesterol are slight and inconstant, thus bearing out the findings of the earlier experiments, and confirming the probability that cholesterol takes no part in the early stages of fat metabolism.

Lecithin (Phosphatides).—In all these experiments as in the earlier ones, lecithin is found to increase in the blood during fat absorption.

In the whole blood the greatest increase noted is in Experiment 1, where the lecithin increases 70 per cent. In the other experiments the increases are all about 30 per cent. The changes in the plasma are much less striking than in the whole blood, varying from 13 per cent in Experiment 1, to none in Experiment 7, and averaging about 10 per cent. The increases in the corpuscles are, on the other hand, most marked, varying from 144 per cent

in Experiment 1 to 55 per cent in Experiment 7, and averaging 83 per cent. These great increases together with the similar increases of the total fatty acids in the corpuscles noted above, are believed to be conclusive evidence that the blood cells absorb the fat from the plasma and transform it into lecithin. Quite in agreement with the above as establishing the relation between the blood cells and lecithin is the recent work of Thiele[18] and of Foà[19] who report the presence in blood of an esterase which decomposes the lecithin of the blood on standing, a change which takes place only in the presence of the corpuscles. Since these enzyme reactions in the living organism are generally regarded as reversible, the *formation* of lecithin in the presence of excess of fat in the blood as above is to be expected. Also, if the reaction were a reversible one, the relation between the fat and the lecithin would be expected to approximate a constant (as indeed has been reported for the blood corpuscles by Mayer and Schaeffer.[20] On the other hand, in view of the constantly changing conditions prevailing in the blood during fat absorption and the limitations of the methods employed for the analyses, it was hardly to be expected that an exactly constant relation between the total fatty acids and the lecithin could be demonstrated during the fat absorption, but there is a rather surprising degree of constancy in most cases as may be seen from Table II.

TABLE II.

Relationship between Total Fatty Acids and Lecithin $\left(\frac{\text{T. F. A.}}{\text{L}}\right)$ *in Whole Blood and Corpuscles.*

No. of experiment.	1		2		3		4		5		6		7	
Period.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.	Whole blood.	Corpuscles.
1	1.6	1.2	1.8	1.4	1.7	1.2	1.7	1.6	1.8	2.1	1.7	2.0	1.6	1.8
2	1.7	1.1	1.7	1.4	1.65	1.1	1.7	1.7	2.0	1.8	1.7	1.7	1.5	1.8
3	1.8	1.2	1.5	1.1	1.7	1.1	1.8	1.6	2.1	1.7	2.0	1.8	1.8	1.5
4	1.5	1.2	1.6	1.1	1.4	0.9	2.1	1.7	2.1	2.3	1.9	2.2	1.8	1.6
5	1.7	1.2	2.3	1.6	1.65	1.0	1.8	1.4	2.4	2 2	2.0	2.1	2.1	2.2

[18] Thiele, F. H., *Biochem. J.*, 1913, vii, 275.

[19] Foà, C., *Arch. ital. biol.*, 1915, lxiii, 239.

[20] Mayer, A., and Schaeffer, G., *J. physiol. et path. gén.*, 1913, xv, 984.

The constancy in the relationship between the fat and the lecithin may also be taken as evidence that all the absorbed fat passes through the lecithin stage and that therefore lecithin is to be regarded as the first stage in fat metabolism.

The hitherto unexplainable anatomical mechanism (thoracic duct) by which the fats are thrown directly into the blood stream and thoroughly mixed with the blood in the heart and lungs before they reach any of the organs of intermediate metabolism (*e.g.*, liver) may be explained on the basis of the above results. Since the first step in the metabolism of the fats takes place in the blood corpuscles it is to be expected that they be given an opportunity to take up as much of the fat as possible before it comes in contact with the organs and tissues.

As to which of the blood cells—red or white—take the predominating part in the lecithin formation, there is no direct information. It has long been known that during fat absorption the white blood cells in the intestinal lymph spaces become loaded with fat and apparently take an active part in the absorption, so that the idea would occur to many that the white cells were responsible for the lecithin increase in the blood. However in view of the fact that they constitute less than 0.5 per cent of the total blood cells it would seem to be physically impossible for them to transform the relatively great amount of fat taken into the blood during a fat meal.

It is quite unlikely, on the other hand, that the lecithin formation is limited to the blood cells. Esterases have been found in many tissues,[21] and it is probable that the circulating fat is removed from the blood stream and worked up by many types of fixed cell in the same way as it is done by the blood cells.

SUMMARY.

Determinations of total fat, lecithin (phosphatides), and cholesterol have been made in whole blood and plasma (and by calculation in the corpuscles) during a series of fat absorption experiments, with the following results.

1. Total fatty acids increase in both plasma and corpuscles but the increase is generally more marked in the corpuscles.

[21] Loevenhart, A. S., *Am. J. Physiol.*, 1901–02, vi, 331.

2. Lecithin increases greatly in the corpuscles but only slightly in the plasma.

3. No definite changes in the quantity of cholesterol were noted.

4. A fairly constant relationship between total fatty acids and lecithin was noted in whole blood and corpuscles.

These results are believed to justify the conclusions: (a) that the blood corpuscles take up the fat from the plasma and transform it into lecithin; (b) that most if not all of the absorbed fat is so transformed; and therefore (c) that lecithin is an intermediate step in the metabolism of the fats.

THE ORIGIN OF THE NITRATES IN THE URINE.

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Nitrates appear to be constant constituents of human urine. Calculated as the potassium salt, human urine contains[1] ordinarily 100 to 200 mg. of nitrate per liter, and occasionally 300 to 400 mg. or more. On normal rations other animals also excrete nitrates in the urine. The question of the origin of the urinary nitrates has not been settled satisfactorily, at least in the case of human urine. Thus the evidence is not conclusive as to whether they are derived only from the food or whether they are formed to some extent by animal tissues. The problem is of peculiar theoretical interest, since the production of an oxidized nitrogenous radicle by animal tissues would be unique. A somewhat extended historical introduction to this rather obscure question seems necessary for a proper appreciation of the experiments described later.

In the oxidation of nitrogenous organic compounds containing nitrogen in amino or imino groupings, one of the first steps in the process seems to be a splitting off of the nitrogen, either directly, as in the deamination of amino-acids and amino purines, or after a hydrolytic cleavage, as in the utilization of polypeptides or tissue protein. The nitrogen thus removed subsequently appears in the urine largely as urea, ammonium salts, or, in the case of birds and reptiles, as uric acid. In the case of amino aromatic bodies, in which the amino group is substituted in the carbocyclic nucleus, the amino group undergoes no change in the animal organism if the nucleus itself remains intact, aside from conversions into acetyl, uramido, or methyl substituted groups. Again, nitrile or cyanide radicles are eliminated from the body as inorganic sulfocyanides.[2]

From such facts as the above, it is evident that the body is unable to derive energy from the nitrogen atom to any great extent, at least, by

[1] Caron, H., *Ann. chim. anal.*, 1912, xvii, 9.

[2] Lang, S., *Arch. exp. Path. u. Pharm.*, 1895, xxxvi, 75.

combining it with oxygen, in marked contrast to the ease with which it oxidizes the carbon and hydrogen atoms. In fact, the organism has a strong reducing action towards nitrogen in an oxidized condition. Thus, nitrobenzene taken *per os* is excreted in the urine in part as *p*-aminophenol, while *o*-nitrophenol appears to be excreted unchanged.[3] Phenylhydroxylamine, as well as its nitroso derivative, has recently been shown by Sieburg[4] to be in part, at least, transformed into *p*-aminophenol and to be excreted in the urine as the lactam of *p*-aminophenol glucuronic acid.

An interesting illustration of the reduction of radicles containing nitrogen in an oxidized condition, is the fate of *m*-nitrobenzaldehyde in the animal organism. Cohn[5] has shown that this compound, when introduced into the rabbit, suffers oxidation of the aldehyde to the carboxyl group, while simultaneously the nitro group is reduced to the amino group, which is acetylated, so that the final product is *m*-acetylaminobenzoic acid. In the body of the dog, however, this transformation does not take place, the final product in this case being urea *m*-nitrohippurate.

Ingested inorganic nitrates are known to be partially and in some cases almost entirely reduced. Thus, Röhmann[6] after giving rabbits 30 to 50 mg. of potassium nitrate in the food was only able to recover 54 to 70 per cent in the urine. Of 500 mg. of potassium nitrate subcutaneously injected, only 55 per cent was excreted in the urine. With dogs even smaller percentages than these were recovered in the urine. Grüter[7] was unable to recover any portion of nitrate doses from the urine of a rabbit either when given *per os* or intravenously. His method of determining nitrates in urine was, however, open to objection. In two experiments on the intravenous injection of nitrates into rabbits, Neuffer[8] was able to recover only 41 per cent in the urine. As was also the case in Röhmann's work, all of the nitrate was not eliminated from the body until 4 or 5 days after the nitrate was given.

Such observations as the above on the destruction of inorganic nitrates by the body have been repeatedly confirmed, though the results are remarkably variable as to the extent of reduction. Thus, Weyl and Citron,[9] in experiments with a dog, were able to recover only 12 per cent of the ingested nitrate in the urine, and with ducks and hens only about 33 per cent; while

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[3] Meyer, E., *Z. physiol. Chem.*, 1906, xlvi, 497.

[4] Sieburg, E., *Z. physiol. Chem.*, 1914, xcii, 331.

[5] Cohn, R., *Z. physiol. Chem.*, 1893, xvii, 274; *Arch. exp. Path. u. Pharm.*, 1905, liii, 435.

[6] Röhmann, F., *Z. physiol. Chem.*, 1881, v, 233.

[7] Grüter, L., Über das Schicksal der salpetersauren Salze im Tierkörper, Dissertation, Würzburg, 1895.

[8] Neuffer, L., Ueber das Verschwinden von Salpetersäure im Stoffwechsel, Dissertation, Würzburg, 1898.

[9] Weyl, T., and Citron, *Arch. path. Anat. u. Physiol.*, 1885, ci, 175. Weyl, T., *ibid.*, 1886, cv, 187.

Gossels[10] was unable to detect any increase in the nitrates of human urine, even after the ingestion of as much as 3 gm. of potassium nitrate. On the other hand, recent work by Grafe and Wintz,[11] and Abderhalden and Hirsch,[12] on dogs and pigs indicates that ingested nitrates may be quantitatively eliminated in the urine in some cases. The greatest destruction of ingested nitrate in these experiments amounted only to 10 or 15 per cent. These discrepancies are probably in large part due to different methods used in the determination of nitrates in the urine, and in part to the use of different species of experimental animals; but after allowing for these factors it seems probable that the extent of the destruction of nitrates taken with the food or injected directly into the blood may vary within wide limits.

The location of the reduction of nitrates in the body has been the subject of many researches.[13] In the course of many investigations on the action of bacteria on nitrates, a subject of great importance to agriculture, it has been shown that many of the bacteria occurring in the intestines of animals are capable of reducing nitrates to nitrites. Thus, *B. coli communis* has the power of reducing nitrates to nitrites,[14] while the occasional production of the symptoms of nitrite poisoning after nitrate ingestion,[15] or after the injection of bismuth subnitrate into the intestine for radiographic investigations,[16] points to the capacity of the intestinal contents of accomplishing the first step in the reduction of nitrates.

That the reducing action of intestinal bacteria on nitrates does not stop at this stage, but may continue to the formation of ammonia and nitrogen gas has also been determined. Thus, Maassen found that, especially in the presence of carbohydrates or polyatomic alcohols, *B. coli communis* was able to destroy nitrates and nitrites entirely in artificial culture media in the course of 2 weeks. Burri and Stutzer[17] showed that horse feces pos-

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[10] Gossels, W., Die Nitrate des Tier- und Pflanzenkörpers, Dissertation, Berlin, 1886.

[11] Grafe, E., and Wintz, H., *Z. physiol. Chem.*, 1913, lxxxvi, 283.

[12] Abderhalden, E., and Hirsch, P., *Z. physiol. Chem.*, 1913, lxxxiv, 189.

[13] In the preparation of this review of the literature, particularly of that portion immediately following, we wish to acknowledge the assistance obtained from an unpublished manuscript of Professor A. P. Mathews on "The Pharmacology of Nitrates and Nitrites."

[14] Maassen, A., *Arb. k. Gsndhtsamte.*, 1902, xviii, 21. Pelz, E., *Centr. Bakt., 1te Abt., Orig.*, 1910, lvii, 1.

[15] Barth, A. F., Toxicologische Untersuchungen über Chilisalpeter, Inaug. Dissertation, Bonn, 1879. Binz, C., and Gerlinger, P., *Arch. int. Pharm. et Thérap.*, 1901, ix, 441.

[16] Böhme, A., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 441. Rautenberg, E., *Berl. klin. Woch.*, 1906, xliii, 1397. Worden, C. B., Sailer, J., Pancoast, H. K., and Davis, G. G., *Univ. Pennsylvania Med. Bull.*, 1906. Zadek, J., *Z. exp. Path. u. Therap.*, 1914, xv, 498.

[17] Burri, R., and Stutzer, A., *Centr. Bakt., 2te Abt.*, 1895, i, 257.

sessed the property of developing free nitrogen from nitrates, a fact which has been confirmed by Grüter.[7,18] The former investigators isolated from manure a denitrifying bacillus which in symbiosis with *B. coli communis* was able to liberate nitrogen gas from nitrate solutions. These denitrifying bacilli seem to be more active in the feces from the herbivora, though Maassen has frequently obtained such bacteria from human feces.

The presence in the intestinal contents, especially of the large intestine, of nitrite-forming and denitrifying bacteria and also the production of nascent hydrogen, which is constantly taking place in the intestines, might well account for the partial destruction of nitrates taken *per os*. It would, however, have no bearing on the partial destruction of nitrates injected directly into the body, subcutaneously or intravenously, especially since it has been shown by Neuffer that in such experiments excretion of nitrates into the intestine with subsequent reduction does not occur. Evidently in such cases, the tissues themselves must exert a reducing action. That the tissues possess strong reducing powers, illustrated by the formation of fats from carbohydrates, the production of hydroxy-acids from keto-acids, the reduction of arsenic to arsenious acid, of the nitro group to the amino group, of substituted hydroxylamines to amino compounds, of iodates to iodides, chlorates to chlorides, and sulfur to hydrogen sulfide, is well established. Similarly, the capacity of organ extracts to reduce nitrates, at least to nitrites, seems fairly well established. Gscheidlen[19] obtained some evidence that the muscle tissue of a frog was able to reduce nitrates to nitrites, and that active muscles were more powerful in this respect than inactive. Mulzer,[20] however, was unable to confirm this result, although he was able to detect a strong reducing action on nitrates by many of the internal organs. Neuffer[8] incubated various tissues and organs of a rabbit for 24 hours with definite amounts of a 1 per cent solution of sodium nitrate and was able to demonstrate a loss of nitrate, especially in the case of liver tissue, and to some extent in the case of muscle tissue and blood. Boiled liver tissue had no demonstrable effect.

The results of Neuffer have been confirmed by Abelous and Gérard.[21] These investigators incubated macerated horse kidney with a solution of potassium nitrate. After 12 to 15 hours the presence of nitrites could be demonstrated by several reactions. The reactions were not obtained if the kidney pulp was previously heated to 100° C. It was found that the reducing substance could be extracted by water or glycerol and that its activity varied with the temperature in a similar manner to enzyme action. Other tissues were tested in a similar manner and were found to have a

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[18] Zuntz, H., *Arch. Anat. u. Physiol., Physiol. Abt.*, 1886, 560.

[19] Gscheidlen, R., *Arch. ges. Physiol.*, 1874, viii, 506.

[20] Mulzer, M., Toxicologische Studien über das Natriumnitrat mit Beziehung auf andere Natriumsalze, Inaug. Dissertation, Würzburg, 1892; *Jahresb. Tierchem.*, 1900, xxx, 101.

[21] Abelous, E., and Gérard, E., *Compt. rend. Acad.*, 1899, cxxix, 56, 164, 1023.

similar action, though in very unequal degree. The liver, kidney, and suprarenal tissues seemed particularly powerful, while muscle and brain tissues were relatively weak in their action. In all their work, bacterial action was excluded by the use of either chloroform, sodium fluoride, or thymol. The results of Abelous and Gérard were later confirmed by Vogelsohn,[22] who also concluded that extracts of liver and kidney tissue contain enzymes capable of converting nitrates into nitrites. In this work, a large part of the nitrite formed had apparently been further reduced, so that it escaped recognition.

In view of the investigations just mentioned, it seems probable that the tissues are able to reduce nitrates to nitrites and possibly to ammonia, though it is evident that under average conditions the nitrite stage must be transient, because the entrance into the adult body, either enterally or parenterally, of considerable amounts of nitrate is only occasionally followed by symptoms of nitrite poisoning. Such symptoms would be produced by very minute amounts of nitrite, the poisonous action of nitrites being extremely potent. The subsequent steps in the reduction of nitrates beyond the nitrite stage is conjectural. Franzen and Löhmann[23] consider exhaustively the possibility of a conversion into ammonia through dihydroxy ammonia and hydroxylamine. It seems probable that ammonia would finally be produced by the tissues rather than free nitrogen, as suggested by Röhmann, though no experimental evidence exists either one way or the other.

While undoubtedly the preponderating tendency in animal metabolism is towards the production and subsequent excretion of unoxidized nitrogen, there is good evidence that under certain conditions nitrogenous radicles may be transformed from a less to a greater oxidized condition. The best evidence on this point concerns the conversion of nitrites to nitrates. Under normal conditions the evidence,[6,24] indicates that nitrites are not excreted in the urine, while nitrates are. Apparently nitrites formed in metabolism from the reduction of nitrates ingested with the food are further reduced in the tissues. Also, when moderate amounts of inorganic nitrates are added to the food or subcutaneously injected into an animal, the fresh urine is free from nitrites. However, from the work of Barth and later of Binz and Gerlinger,[15] it appears that when excessive amounts of inorganic nitrates are given to an animal, the fresh urine may respond to nitrite tests and may contain even considerable amounts of nitrite.

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[22] Vogelsohn, A., Ueber die Einwirkung von Organextrakten auf Nitrate und Nitrite, Inaug. Dissertation, Bern, 1907; *Jahresb. Tierchem.*, 1908, xxxviii, 874. Schönbein, C. F., *Z. Biol.*, 1867, iii, 334. Stepanow, A., *Arch. exp. Path. u. Pharm.*, 1902, xlvii, 411. Thunberg, T., *Ergebn. Physiol.*, 1911, xi, 328.

[23] Franzen, H., and Löhmann, E., *Z. physiol. Chem.*, 1909, lxiii, 100. Neuberg, C., and Welde, E., *Biochem. Z.*, 1914, lxvii, 18.

[24] Schönbein, *J. prakt. Chem.*, 1864, xciii, 463.

The excellent work of Röhmann in 1881 proved that even after subcutaneous injection of moderate amounts of sodium nitrite into rabbits the fresh urine of the same and subsequent days failed to respond to the nitrite test. On the other hand, after the injection of 100 to 500 mg. of this salt, the urine, which previously was nitrate-free,[25] was found to contain nitrates for 2 or 3 days following the nitrite injection. However, only 36 to 43 per cent of the quantity of nitrite injected could be accounted for by the quantity of nitrate appearing in the urine, the remainder apparently suffering reduction in the body.

These observations of Röhmann have been confirmed by Mulzer[20] and Atkinson.[26] Mulzer, after injecting rabbits with sodium nitrite, was unable to detect nitrites in the fresh urine. On standing, however, a nitrite reaction developed, obviously from preformed nitrates. Atkinson administered sodium nitrite to rabbits, and very rarely found nitrites in the urine, even after fatal doses had been given. Nitrates, however, were always present, though the animal was on a diet which produced no excretion of nitrates in the urine. In agreement with Röhmann, Atkinson was able to recover only a part of the administered nitrite as nitrate—in the one experiment cited, 47 per cent. In no case were nitrates or nitrites found in the feces. In the case of dogs, nitrites could readily be detected in the urine for 24 to 36 hours after the subcutaneous injection of 130 to 194 mg. of sodium nitrite. The more vigorous the dog was, the less readily was the nitrite detected. The same circumstance was found to hold in the case of man, men in vigorous health rarely having nitrite reactions in their urine, even after considerable doses, while patients in bed give it comparatively readily.

Other instances of the oxidation of nitrogen have been reported, though they have never been confirmed. We refer to the early experiments of Bence-Jones[27] on the oxidation of ammonium salts to nitric acid. This investigator seems to have obtained clear-cut evidence with human subjects in support of his conclusions, but Wulffius,[28] using Bence-Jones' analytical methods with a man as subject, and Weyl and Citron,[9] experimenting on dogs, were unable to confirm them. Ville and Mestrezat,[29] in their report of investigations on the origin of the nitrites of the saliva, state that pure parotid and submaxillary saliva contains nitrates, which are subsequently reduced to nitrites in the mouth cavity. They also state that the nitrate content of saliva may be increased by the ingestion of capsules of sodium nitrate, sodium nitrite, or ammonium salts. No details of the experimental technique or of any quantitative results are

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[25] On a ration of milk and wheat bread.

[26] Atkinson, G. A., *J. Anat. and Physiol.*, 1887–88, xxii, 351.

[27] Bence-Jones, H., *Phil. Tr. Roy. Soc. London*, 1851, cxli, 399.

[28] Wulffius, E., Ueber den Nachweis von Salpetersäure im Harn, Inaug. Dissertation, Dorpat, 1861.

[29] Ville, J., and Mestrezat, W., *Compt. rend. Soc. biol.*, 1907. lxiii, 231.

reported. While the production of nitrates from nitrites by the salivary glands is in line with the work of Röhmann, Mulzer, and Atkinson, the question of the oxidation of ammonium salts to nitrates is in need of further confirmation.

Recently Mazé[30] claims to have proven the production of nitrous acid by living animal cells. Blood corpuscles of a rabbit were separated aseptically from the plasma by centrifugation, washed three times in physiological (0.9 per cent) salt solution, and finally suspended in this solution so as to give an emulsion of the form elements of blood five to ten times more dilute than blood itself. In this medium the corpuscles remained alive for several days. If hemolysis was not too rapid, Mazé was able to detect nitrous acid by the iodide test in less than 48 hours at ordinary temperatures and in 3 to 4 days at 10°. The reaction was neither constant nor regular, apparently depending on unknown factors. The reaction disappeared as soon as hemolysis was well under way. Mazé expresses the opinion from the results of this and other experiments on bacteria and plant juices that it is probable that nitrous acid exists in the cell in combination. The report of this work may be criticized for failure to specify all experimental details, such as the precautions taken to exclude bacterial action. It is also unfortunate that some more specific tests than the iodide test were not employed for the detection of nitrous acid, while the inconstant and variable results obtained cannot but detract from the confidence in the particular interpretation given to them by Mazé. This investigator also claims to have proved the presence of nitrous acid in fresh urine under normal conditions.

The possibility that the body is able to oxidize ammonium salts or amino bodies to nitrates has been tested by several investigations on the origin of nitrates in the urine. Nitrates constantly occur in the urine of animals on normal rations. The wide distribution of nitrates in food materials, especially in fresh vegetables,[31] has led to the common supposition, expressed in text-books on physiological chemistry, that the urinary nitrates have their sole origin in the food consumed. There is good evidence substantiating this view. Röhmann was unable to detect nitrates in the urine of dogs fed exclusively on meat or in the urine of rabbits fed exclusively on milk and wheat bread. The work of Röhmann seems reliable and is extensively quoted. It was later completely confirmed by Weyl and Citron.[32]

On the other hand, the results with human subjects seem to bear another

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[30] Mazé, *Compt. rend. Acad.*, 1911, clii, 1624; 1911, cliii, 357. In his second paper Mazé mentions a confirmation of Bence-Jones' work by Müntz, in the nature of a verbal communication. An exhaustive review of the literature, however, has failed to reveal any published report of such work.

[31] Richardson, W. D., *J. Am. Chem. Soc.*, 1907, xxix, 1757.

[32] Weyl and Citron, *Arch. path. Anat. u. Physiol.*, 1885, ci, 178.

interpretation. Thus, Wulffius,[28] experimenting on himself, eliminated all vegetables from his diet and used distilled water in the preparation of all food except of the bread used. Upon distilling the concentrated urine with sulfuric acid and testing the distillate with the iodide method for nitrous acid, it was found that as the experiment progressed the above reaction became less and less marked, but did not disappear. Weyl and Citron[33] found that men on an exclusive meat diet still excreted nitrates in the urine, equivalent to about 35 mg. of N_2O_5 per liter. The details of these experiments are not reported. These investigators report an analysis on the urine of a patient with progressive paralysis and beginning decubitus. The patient received only milk, meat broth, and eggs, foods practically nitrate-free. The urine was found to contain about 63 mg. of N_2O_5 per liter. Gossels[10] in Weyl's laboratory, on a vegetable-free diet excreted from 5 to 8 mg. of N_2O_5 per day. The evidence cited seems to indicate that with man the excretion of nitrates in the urine is not all derived from nitrates ingested with the food. It at least furnishes the basis of a fair suspicion that the nitrate metabolism of man may be qualitatively different from that of the dog and the rabbit.

Some experimental results obtained in this laboratory in 1907–08 on men afford strong support to the view that the human organism is able to produce nitrates from unoxidized nitrogen. In the course of an extensive metabolism experiment on 24 men, the results of which will be published in detail elsewhere, the following facts as regards the metabolism of nitrates were determined.

1. For a period of 220 days, twelve of the subjects received a diet consisting largely of foods containing no nitrates or only mere traces. While the nitrate analyses on the different foods used were not as complete as might be desired, such as they were they would indicate that the nitrate intake per man per day was probably less than the equivalent of 25 mg. of potassium nitrate. During the 220 days on this diet, the 4 day composite collections of urines were occasionally analyzed for nitrates by the Schulze method, over 180 analyses being made on the urines of the twelve men. The average daily excretion was about 82 mg. of potassium nitrate. Marked individual differences were noted. Two men consistently excreted from 100 to 150 mg. per day. Two others on the same diet excreted only 30 to 35 mg. per day. However, from the data at hand it appeared highly probable that most of the twelve men were consistently excreting more nitrate than they

[33] Weyl and Citron, *Arch. path. Anat. u. Physiol.*, 1885, ci, 187.

were ingesting, and in many cases from three to six times the amount ingested.

2. Twelve other men during a fore-period of 92 days, while consuming the same diet as the first group of men, excreted quantities of nitrate comparable to the excretions noted above. During an immediately following period of 96 days they received the same diet as in the fore-period with the addition of variable amounts of potassium nitrate, from 80 to 124 mg. per day. The increased nitrate intakes in all cases increased the nitrate excretions in the urine. With the one exception noted in the following paragraph, the increases in the daily excretion of urinary nitrates were less than the increases in the daily intake of nitrates, indicating a partial destruction of the nitrate added to the food. An interesting relation was discovered between the nitrate content of the urine and the increase in the nitrate content on the addition of nitrates to the food consumed, such that, in general, the greater the former, the greater the latter. This would indicate that the differences among the subjects as regards the quantity of nitrates excreted per day in a measure indicated differences in the ability to destroy ingested nitrates, and probably also differences in the intensity of nitrate production in the body.

3. The exceptional behavior of two of the subjects of the latter group of twelve men is worthy of special mention. One subject on the nitrate-poor diet of the fore-period excreted an average of 267 mg. of nitrate (expressed as KNO_3) in the urine per day, this figure being an average of seven analyses. During a subsequent period of 72 days, when 93 mg. of KNO_3 were added to his food per day, his urinary excretion averaged 368 mg. per day (sixteen analyses). During the next 56 days, 124 mg. of KNO_3 were given per day. His urinary excretion rose from an average of 368 mg. to an average of 490 mg. per day (thirteen analyses). From such data there seems to be little doubt that this man was producing nitrates in the body and that apparently the introduction of nitrate with the food stimulated the production of nitrate in the body. In marked contrast to this subject, a second subject, throughout the 128 days during which nitrate was added to the diet, excreted less nitrate in the urine than the amount of nitrate added. The quantity of nitrate excreted per

day in the urine averaged only 53 per cent of the quantity of nitrate added per day to the food. The first subject averaged about 78 kg. in weight, and the second about 57 kg. However, considering all the subjects of the experiment, no close correlation apparently existed between body weight and the excretion of nitrates in the urine, though in general the men of large body weight excreted the largest quantities of nitrate, while the men excreting the smallest quantities of nitrate were in general below the average in weight.

4. At no time were nitrates or nitrites discovered in the feces, nor were nitrites ever detected in the fresh urines.

The results of this experiment can apparently bear only one interpretation; *i.e.*, that the nitrates in human urine are not derived entirely from the nitrates in the food, the presumption being that the body is able to form nitrates from unoxidized nitrogenous radicles. However, there is one weak point in the evidence, and that is that the nitrate intake was not determined consistently or completely throughout the experiment. The foods were analyzed occasionally in the condition in which they were served, generally with negative results. It was therefore considered advisable to undertake experiments in which the nitrate intake would be reduced to as low a level as practicable and in which the nitrate balance would be accurately determined. These experiments will now be considered.

Methods.

In all of our work we used a modification of the Schulze method,[34] in which the nitrates are reduced to nitric oxide by ferrous chloride and hydrochloric acid, the nitric oxide evolved is collected over concentrated alkali, and its volume measured. The only important modifications we have made in this method may be summarized as follows.

1. The decomposition of the nitrates was effected in a 100 cc. Jena round bottom flask, which was heated in an oil bath kept at a temperature of about 155°C. This method of heating

[34] For a good description of the original method see Tiemann-Gärtner's Handbuch der Untersuchung des Wasser, etc., Braunschweig, 4th edition, 1895, 154, 176.

insures a steady decomposition, and the operation conducted in this way does not require as much attention as when a free flame is used.

2. The air was removed from the apparatus by 10 or 15 minutes' boiling of the reagents,[35] after which the solution to be analyzed was introduced gradually into the flask from a glass separatory funnel. In the Schulze method the unknown solution is boiled in the decomposition flask until the air is removed, after which the reagents are introduced. We have adopted the former procedure, since the air may be more quickly expelled by boiling the reagents, while the final results are equally reliable.

3. In determining the amount of nitric oxide in the gas collected its volume was measured before and after absorption with an alkaline permanganate solution, according to the procedure of Van Slyke in removing the nitric oxide from the nitrogen obtained in his method for the determination of aliphatic amino groups.[36] This solution contains 50 gm. of potassium permanganate and 25 gm. of potassium hydroxide per liter. In carrying out the absorption, the gas measuring tube in which the NO was collected is connected, while its lower end is still immersed in the 20 per cent caustic alkali used in our work, with the gas burette of the Van Slyke apparatus by a glass capillary tube and rubber tubing. The Van Slyke burette, the leveling bulb connected with it, and the tubes connecting the burette with the gas tube are filled with a 7 per cent[37] solution of Greenbank's alkali. By opening the stop-cock at the upper end of the gas tube and that giving admission to the gas burette, and by lowering the leveling bulb, the gas is drawn over into the burette. The burette stop-cock is closed as soon as the gas is over so as to prevent unnecessary mixing of the two alkali solutions. After standing in contact with the 7 per cent alkali for 10 minutes, the

[35] The reagents consisted of 15 cc. of a solution of ferrous chloride (prepared by dissolving 500 gm. of clean iron nails in 3 liters of concentrated chemically pure HCl and filtering through sand over glass wool) and 10 cc. of HCl (sp. gr. 1.10).

[36] Van Slyke, D. D., *J. Biol. Chem.*, 1911, ix, 186.

[37] This strength alkali was chosen simply for convenience in calculating the water vapor pressure corrections. The only table readily available for calculating this correction was one in Biedermann's Chemiker-Kalendar for 1914 for alkali of 7 per cent strength.

gas is measured by proper adjustment of the leveling bulb, and is then forced into the Hempel pipette containing the alkaline permanganate solution. The pipette is then shaken by a small electric motor for about 5 minutes and the residue drawn over into the burette and its volume measured. The absorption is then repeated to determine whether it is complete. The difference in volume between the original measurement and the final measurement of the residual gas not absorbed by the alkaline permanganate solution, is a measure of the nitric oxide obtained in the analysis.

4. In analyzing the foods, it was generally found advisable, especially with the starchy foods, to extract them thoroughly with 75 per cent alcohol, checking the completeness of the extraction with the diphenylamine reaction. In some cases a water extract was made. The extract was then made alkaline with sodium hydroxide and evaporated on the water bath. If during the evaporation a coagulum formed it was filtered off. The evaporation was continued until a volume of about 15 cc. was obtained. The extract was then ready to be introduced into the nitrate apparatus. The size of sample taken was ordinarily 30 to 50 gm. In case of foods known to contain only traces of nitrate, this sample at times weighed as high as 100 gm. In analyzing milk, 200 cc. were taken and the protein was precipitated with trichloroacetic acid, filtered off, and washed thoroughly with water. The filtrate was then evaporated on the water bath to a small volume and was then ready for analysis. All analyses were run in duplicate or triplicate.

5. In the analysis of urine, 200 cc. portions were taken in most cases, except with the urines in the experiments on rats. The samples were then made alkaline, unless already in that condition, and evaporated to about 25 cc. on the water bath. They were then treated with 75 cc. of 75 per cent alcohol, filtered, the precipitate was carefully washed with 75 per cent alcohol, and the filtrate concentrated on the water bath to about 10 to 15 cc. They were then ready for analysis and gave no trouble by foaming. These analyses were also run in duplicate or triplicate whenever possible. With the rat urines it was necessary to take the entire collection for a single analysis.

The Accuracy of the Nitrate Method.

The accuracy of all methods based on the reduction of nitric acid to nitric oxide by ferrous chloride, in the presence of organic matter, has been the subject of considerable investigation. These methods have proven to be especially valuable in the analysis of soils, urine, animal fertilizer, and a wide variety of plant and animal material. The results of most analysts have indicated the trustworthiness of the methods in the presence of urea, ammonium salts, proteins and protein derivatives, organic acids, etc., and, in general, good results have been reported on the analysis of urine and other materials containing organic substances.

However, some results have been reported which indicate a disturbing effect of urea and ammonium salts in particular. Pfeiffer and Thurmann, and Pfeiffer[38] have concluded from a large number of analyses that the presence of urea and ammonium salts reduces the theoretical yield of nitric oxide. On the other hand, Seydel and Wichers[39] conclude that urea increases the theoretical yield of nitric oxide. Opposed to the conclusions of all these analysts stands the excellent work of Warington,[40] Liechti and Ritter,[38] and the analyses reported in Tiemann-Gärtner's Handbuch[34] (page 221), according to which urea and ammonium salts exert no demonstrable effect on the reduction of nitrates to nitric oxide.

Working with pure nitrate solutions (samples containing 50 to 150 mg. of KNO_3), we have found that from 95 to 100 per cent of the theoretical quantities of nitric oxide may be recovered under the most favorable conditions, using the method outlined above.[41] As a general rule, in agreement with other investigators,

[38] Pfeiffer, T., and Thurmann, H., *Landw. Vers.-Stat.*, 1895, xlvi, 1. Pfeiffer, T., *Z. anal. Chem.*, 1903, xlii, 612. For a good review of the literature on this point see Liechti, P., and Ritter, E., *Z. anal. Chem.*, 1903, xlii, 205.

[39] Seydel, S., and Wichers, L., *Z. angew. Chem.*, 1911, xxiv, 2046.

[40] Warington, R., *J. Chem. Soc.*, 1880, xxxvii, 468; 1882, xli, 345; also cited by Hintz, E., *Z. anal. Chem.*, 1884, xxiii, 545.

[41] In this work on pure nitrate solutions, the gas collected was not treated with the permanganate solution but was measured directly. It is therefore comparable to the results of other investigators.

we have obtained more satisfactory results with the larger quantities of nitrate. The presence of a large excess of urea (twenty to thirty times the weight of nitrate taken) appeared to exert no definite effect on the volume of gas obtained, though in general it was more difficult to obtain good duplicates in the presence of this substance. We did not investigate the influence of ammonium salts, since our method of preparing the food and urine samples for analysis involved the decomposition of all ammonium salts present.

In our first analyses of human urine, four samples were taken from each collection, three were analyzed for nitrates directly, and the fourth after the addition of a definite weight of pure potassium nitrate. From the results obtained with nineteen samples of urine to which from 22 to 194 mg. of potassium nitrate were added, it was found that an average of 92.4 per cent of the added nitrate could be accounted for by the quantity of NO gas obtained. With 10 samples of urine, each consisting of 200 cc., to each of which 10.111 mg. of KNO_3 were added, an average of 93.2 per cent of the added nitrate was recovered.

The lower percentage of added nitrate recovered from urine as compared with distilled water solutions is in agreement with the results of Liechti and Ritter. These analysts were able to recover an average of 98.2 per cent of the nitrate in water solution (fourteen analyses), and an average of 94.8 per cent in urine solution (eleven analyses). In the method used by Liechti and Ritter, the total gas collected not absorbed by alkali was measured and calculated as NO. This fact may account for the slightly larger average recovery of nitrate added to urine of known nitrate content.[42] It is evidently a fact that the method is not as satisfactory in the analysis of urine as in the analysis of pure nitrate solutions, but to what constituent of urine the disturbing effect is due cannot at present be said. It was also our experi-

[42] The average residual gas unabsorbed by the alkaline permanganate solution for the urine analyses was 0.25 cc. (160 analyses). The amount of residual gas was not obviously correlated with the amount of total gas obtained. In the food analyses larger residues were obtained, *i.e.*, about 0.50 cc., while in the case of the analysis of milk, high values were always obtained, averaging about 0.80 cc.

ence that it is more difficult to obtain satisfactory duplicates with urines than with pure nitrate solutions. Our analyses of human urine and pig's urine were all run in triplicate, and if good agreement was not obtained, a second analysis in duplicate or triplicate was run whenever possible. In this connection, we determined by repeated tests that thymol and refrigeration will preserve urine for at least 5 days at constant nitrate content.

TABLE I.

Analytical Results on the Recovery of Potassium Nitrate Added to Wheat Bread and Boiled Potato.

Food used.	KNO_3 added.	KNO_3 recovered.	Recovery.
	mg.	*mg.*	*per cent*
25 gm. bread	22.0	17.0	77.3
25 " "	24.1	19.2	79.7
25 " "	56.2	46.5	82.7
25 " "	53.4	37.5	70.2
25 " "	81.9	62.1	75.8
25 " "	78.8	61.6	78.2
25 " "	132.2	99.7	75.4
25 " "	142.6	90.8	63.7
			Average, 75.4
35 gm. potato	24.2	20.2	83.5
35 " "	23.7	23.5	99.2
35 " "	142.5	110.0	77.2
35 " "	131.0	111.7	85.3
			Average, 86.3

In testing the method for the analysis of nitrates in foods the results obtained were not as satisfactory as those obtained from the tests with urine. In Table I are given the data resulting from adding definite amounts of potassium nitrate to bread and boiled potatoes, extracting in the usual way, and determining the nitrate in the concentrated and filtered extract. The extraction of the foods was continued until the diphenylamine test applied to the extracts was negative.

The figures given in the third column of the table were obtained after due allowance was made for the nitrate content of the foods

used.[43] Evidently better results were obtained with the potatoes than with the bread.[44]

These tests indicate that a smaller percentage of the nitrate added to foods is recovered by the nitrate method used than in the case of the addition of nitrate to urine. This is very probably due to the reduction of some of the nitrate by the soluble carbohydrates of foods. Thus, Warington[40] found that the addition of sugar to a nitrate solution lowered the percentage recovery of the nitrate, a result also noted by Schloesing.[45]

However, this reducing action of carbohydrates on nitrates may be more than compensated by the production of other gases in the decomposition flask, among which Warington has found carbon monoxide as an important constituent. Warington found that, in the presence of carbohydrates, much larger volumes of residual gases, unabsorbable by soda solution or a solution of a ferrous salt, resulted than in their absence. Our experience has abundantly confirmed the production of gases from the action of carbohydrates on ferrous chloride and hydrochloric acid. Thus three 10 gm. samples of sucrose were analyzed by the method outlined in this paper, with the production of 0.87, 1.02, and 0.81 cc. of gas. Upon shaking this gas in the Hempel pipette with the alkaline permanganate solution, these volumes were gradually reduced to 0.80, 0.91, and 0.69 cc., respectively.

The reduction in volume noted was probably due to the oxidation of the CO gas present and the retention of the CO_2 in the pipette. To test the relative absorption of NO and CO by the alkaline permanganate solution the following experiment was undertaken. Nitric oxide gas was produced by the action of ferrous chloride and hydrochloric acid on pure KNO_3. This gas

[43] The bread analyzed 0.196 mg. KNO_3 per 25 gm., or 0.0008 per cent. The potato gave 2.45 mg. KNO_3 per 35 gm., or 0.007 per cent.

[44] An interesting though unexplained observation was made in evaporating the water extracts of the bread samples on the water bath after they had been made alkaline. At first they were all of a light yellow color, but as digestion proceeded, they all darkened, though unequally. On several hours' heating, those samples containing no added nitrate were dark brown in color, those containing the most added nitrate were lightest in color, while the gradations in color between these extremes were roughly proportional to the quantity of added nitrate.

[45] Schloesing, T., *Ann. chim. phys.*, 1854, series 3, xl, 479.

was shaken for successive 30 second intervals in the Hempel pipette with alkaline permanganate solution and the absorption followed by measuring the residual gas between these intervals. The results of three experiments are given below:

	I.	II.	III.
	cc.	cc.	cc.
Original volume of NO	28.50	39.70	31.40
Volume after 30 seconds' treatment	0.25	1.00	0.65
" " 60 " "	0.20	0.95	0.55
" " 90 " "	0.15	0.90	0.50
" " 120 " "	0.15	0.90	0.50

Evidently the NO is rapidly absorbed, complete absorption resulting after 90 seconds' shaking with the absorbent. The same sort of a test was run on CO, obtained by the action of H_2SO_4 on oxalic acid and collection of the gas over alkali. The intervals of shaking were in this case 4 minutes:

	I.	II.
	cc.	cc.
Original volume of CO	38.05	8.87
Volume after 4 min. treatment	31.20	7.31
" " 8 " "	22.70	5.95
" " 12 " "	15.50	4.20
" " 16 " "	7.80	3.57
" " 20 " "	4.80	2.50
" " 24 " "	3.10	2.08
" " 28 " "	2.50	1.76
" " 32 " "	2.00	1.57

This gradual absorption continued until at the end of 52 minutes the first volume was reduced to 1.30 cc., and at the end of 68 minutes the second volume was reduced to 1.05 cc.

It would seem, therefore, that the Schulze method for the determination of nitrates would tend to give too high results in the analysis of foods, especially foods containing much starch or sugars and small amounts of nitrates, and in particular would indicate a much wider distribution of nitrates in food materials than actually exists. Thus, in the experiments on pigs, reported

below, the three foods used when analyzed by the Schulze method gave results indicating a fairly considerable content of nitrate, though a color test for nitrates in the original foods and a test for NO in the gas produced by the action of ferrous chloride and hydrochloric acid, gave entirely negative results.

At times the nitrate determinations on the foods used were extremely erratic. Thus, one sample of cheese on toast, a food used in the first experiment, gave the extreme value of 0.0931 per cent of nitrate as KNO_3, while a repetition of the determination gave 0.00019 per cent, a value consistent with the results obtained on the bread and cheese analyzed separately. Such impossible values as were very occasionally obtained were possibly due to an abnormal production of carbon monoxide. In every case before discarding these data other analyses were made to make sure that they were in fact erratic.

Experiments on Men.

The first subject (H. H. M.) whose nitrate balance was determined was a man about 30 years of age and 68.5 kg. body weight. During the course of the experiment this subject was performing office and laboratory work and was taking his meals at home. From July 20 to 24 (1915) inclusive, preliminary observations were made on the daily excretion of nitrates in the urine. While the subject was on an unrestricted diet, the total quantities of nitrates excreted per day in the urine, expressed as mg. of KNO_3, for these 5 days, and the total volumes of urine per day, were:

	Potassium nitrate.	Urine.
	mg.	*cc.*
July 20	141	1,815
" 21	279	1,475
" 22	98	1,870
" 23	85	1,360
" 24	632	1,390

The irregularity of the excretion depended upon the variable quantity of fresh vegetables eaten. Thus, the high value for July 24 was due to a liberal consumption of beets, which are known to possess a very high content of nitrates.

From July 30 to Aug. 12, inclusive, the subject confined himself to a diet free from fresh vegetables, and consisting largely of potatoes, milk, bread, wheat cakes, crackers, meat, and eggs. During the last 7 or 8 days of this period, potatoes were omitted from the diet, since they were found to contain appreciable quantities of nitrates. The daily excretions of nitrates in the urine were:

	Potassium nitrate.	Urine.
	mg.	*cc.*
July 30	146	1,400
" 31	100	980
Aug. 1	133	1,130
" 2	101	1,190
" 3	119	1,060
" 4	91	1,240
" 5	81	1,215
" 6	68	1,065
" 7	71	1,240
" 8	80	1,010
" 9	67	1,080
" 10	62	1,485
" 11	74	1,400
" 12	49	1,435

It is thus seen that the restricted diet resulted in a lowered excretion of nitrates, which was fairly constant for the last week or so of the period.

During the 4 days from Aug. 13 to 16, inclusive, the subject was on a weighed diet. A sample of all articles of food eaten was reserved for analysis. The water drunk was mainly distilled water, though small amounts of city water were used also. The latter contained small quantities of nitrates, equivalent on an average to about 1 mg. of potassium nitrate per liter.

The food consumed per day during the 4 day period was as follows:

Aug. 13	Wheat cakes, 251 gm.	Beefsteak, 140 gm.
	Milk, 1,500 cc.	Cheese on toast, 157 gm.
	Eggs, 1	Bread, 168 gm.
" 14	Wheat cakes, 309 gm.	Lamb chops, 117 gm.
	Milk, 1,250 cc.	Bread, 179 gm.
	Eggs, 2	Cheese, 44 gm.

Aug. 15	Wheat cakes, 287 gm. Milk, 850 cc. Eggs, 2	Cheese on toast, 226 gm. Bread, 263 gm.
" 16	Eggs, 3 Milk, 1,000 cc.	Bread, 316 gm.

The sugar and butter used were not weighed, nor were small amounts of gravy eaten on Aug. 13 and 14. The bread was eaten fresh or toasted. The meat was not analyzed, but was assumed to be nitrate-free in accordance with the analyses of other investigators.

The nitrate balance of Subject H. H. M. during this period is given in Table II.

TABLE II.

Nitrate Balance of Subject H. H. M.

Date.	Volume of urine.	Nitrate values expressed as mg. of KNO_3.		
		Nitrate intake.	Nitrate in urine.	Nitrate balance.
	cc.			
Aug. 13	1,315	13	61	−48
" 14	1,180	13	51	−38
" 15	917	12	64	−52
" 16	1,175	11	60	−49
Average		12	59	−47

From these data it appears that the subject was excreting in the urine, in a fairly constant manner, about five times as much nitrate as he was ingesting.

The second subject (H. A. S.) was 23 years of age and weighed about 64 kg. For 8 to 10 hours a day this subject was working in the laboratory. On July 21, a day's collection of urine was found to contain 345 mg. of nitrate calculated as KNO_3. The diet at this time was unrestricted and included the usual amount of fresh vegetables. From Aug. 17 to 30, inclusive, the subject was on a weighed diet, consisting as far as possible of foods of low nitrate content, such as shredded wheat biscuit, corn-starch pudding, milk, eggs, and meat. The food intake during this 2 week period is given in Table III.

TABLE III.

Food Intake in the First Experiment with Subject H. A. S. Weights of Solid Foods Expressed in Gm. Milk Intake Expressed in Cc.

Food.	Aug. 18	19	20	21†	22	23	24	25	26	27	28‡	29	30§
Shredded wheat biscuit	112	84	84	84	70	56	112	140	112	84	70	28	28
Whole wheat bread	185	220	165	177	196	173	268	69	181	295			
White bread								221	57		235	260	320
Whole milk	300	225	225	225	100	150	300	375	300	1,172	1,335	75	75
Buttermilk	2,000	1,673	1,973	2,073	1,700	1,847	900	1,700	1,373				
Eggs*	2	2	2	1	1	1	3	2	2	4	5	3	2
Corn-starch pudding	76	119	111	77	352	320		150					
Beef loaf	164			129	126	76							
Other meat		110	90				87	90	73	64		150	
Cake										20		80	

* The consumption of eggs is indicated by the number eaten.

† On this day a dish of ice-cream was eaten. It was not weighed nor was a sample analyzed.

‡ On Aug. 28 there was an extra food consumption of one bar of Hershey's chocolate and one dish of Krumbles.

§ On Aug. 30, besides the foods given in the table, there were consumed 150 gm. of potatoes, 150 gm. of macaroni and cheese, and an unweighed amount of apple butter.

All foods eaten during this period were sampled and analyzed with the exception of the meat other than beef loaf, the cake, and the extra foods mentioned in the foot-notes to the table. Therefore, the nitrate intake for the last 4 days of the period was only approximately determined.

On Aug. 25, 986 mg. of KNO_3 were taken with the food, being distributed among the three meals. In Table IV are given the data of the intake of nitrate (figured as KNO_3), the excretion in the urine, the daily volume of urine, and the apparent nitrate balance.

During this 2 week period the subject was evidently excreting much more nitrate than he was ingesting, generally four to five times as much, and on some days even a greater ratio than this existed. After the ingestion of the potassium nitrate added to the food on Aug. 25, apparently only 60 to 65 per cent was recovered in the urine.

A second experiment on H. A. S. was run from Sept. 24 to Oct. 2, inclusive. During this 9 day period the food intake was under much better control than in the first experiment, being restricted

TABLE IV.

Nitrate Balance in the First Experiment on Subject H. A. S.

Date.	Volume of urine.	Nitrate values expressed as mg. of KNO_3.		
		Nitrate intake.	Nitrate in urine.	Nitrate balance.
	cc.			
Aug. 17	845		57	
" 18	1,545	22	66	−44
" 19	1,125	20	73	−53
" 20	1,475	21	88	−77
" 21	1,615	21	108	−87
" 22	790	20	58	−38
" 23	900	20	79	−59
" 24	820	16	83	−67
" 25	1,515	1,011	588	+423
" 26	1,465	19	169	−150
" 27	1,280	15	108	−93
" 28	1,095	17	120	−103
" 29	1,360	10	154	−144
" 30	2,310	12	90	−78

to eggs, shredded wheat biscuit, corn-starch pudding,[46] milk, and sugar. All foods were carefully weighed out and analyzed. The food intake is given in Table V.

TABLE V.

Food Intake in the Second Experiment on Subject H. A. S.

Food	Sept. 24	25	26	27	28	29	30	Oct. 1	2
Shredded wheat biscuit, gm.	280	218	189	273	276	224	258	316	175
Eggs	2	2	2	0	0	0	0	0	4
Corn-starch pudding, gm.	250	531	210	510	627	955	975	977	750
Milk, cc.	1,184	1,178	946	1,419	1,419	1,419	1,419	1,892	1,146
Sugar, gm.	75	80	55	75	85	89	90	90	60

On Sept. 26 approximately 1 liter of city water was consumed, and on Sept. 30, 200 cc. of city water were taken. Otherwise

[46] The corn-starch pudding was made according to the following recipe: 1 quart of milk, 65 gm. of corn-starch, 3 eggs, seasoned with chemically pure NaCl.

only distilled water was used. On Sept. 22 and 23 the food intake was restricted so that all fresh vegetables were excluded from the diet. Also the urine was collected on these days.

In Table VI are given the data on the nitrate balance during this period, and also the daily volumes of urine.

TABLE VI.

Nitrate Balance in the Second Experiment on Subject H. A. S.

Date.	Volume of urine.	Nitrate values expressed as mg. of KNO_3.		
		Nitrate intake.	Nitrate in urine.	Nitrate balance.
	cc.			
Sept. 22	745		145	
" 23	978		146	
" 24	670	9.4	65	−56
" 25	786	10.6	89	−78
" 26	674	7.6	63	−55
" 27	830	11.5	62	−51
" 28	825	12.1	60	−48
" 29	1,045	13.7	62	−48
" 30	1,300	13.9	51	−37
Oct. 1	1,326	16.8	73	−56
" 2	845	12.1	92	−80

TABLE VII.

Nitrate Content of Foods Used.
Nitrate Values Expressed as Percentage of KNO_3.

Food.	KNO_3	Experiment.	Food.	KNO_3	Experiment.
	per cent			*per cent*	
Wheat cakes	0.00090	H. H. M.	Bread (whole wheat)	0.0023	H. A. S.
Eggs	0.00061*	H. H. M. and H. A. S.	Bread (white)	0.0033	H. A. S.
Bread (white)	0.0017	H. H. M.	Milk	5.6 mg. KNO_3 per l	H. A. S.
Milk	4.9 mg. KNO_3 per l	H. H. M.	Corn-starch pudding	0.00053	H. A. S.
Cheese on toast	0.00019	H. H. M.	Buttermilk	7.2 mg. KNO_3 per l	H. A. S.
Cheese	0.0011	H. H. M.	Beef loaf†	0.00022	H. A. S.
Potato	0.00023	H. A. S.			
Shredded wheat biscuit	0.00033	H. A. S.			

* This is equivalent to 0.28 mg. KNO_3 per egg. It was considered sufficiently accurate in this work to use this figure applied to the number of eggs consumed.

† Made from beef, eggs, and crackers.

In this experiment, as in the preceding two, the nitrate excretion in the urine is several times larger than the intake.

The nitrate contents of the foods used in the above three experiments are given in Table VII.

Experiments on Rats.

Experiments were run on four adult albino rats to determine the nitrate balance, from Aug. 24 to Sept. 10, inclusive. For several weeks previous to this period, and during the experimental period, the rats were kept upon a ration consisting of the following food materials:

	per cent
Whole milk powder	54
Corn-starch	18
Lard	28

Upon the analysis of 10 gm. portions of this ration it was found to contain nitrate equivalent to 0.000166 per cent of KNO_3.

The rats were each kept in a round galvanized iron cage, 8 inches in diameter and 6 inches high. These cages were firmly supported in short-stem glass funnels with a maximum diameter of 10.5 inches, which in turn were supported in a wooden frame. Beneath each funnel was placed a wide-mouthed bottle with a capacity of about 10 ounces, for the collection of the urine. Both funnels and bottles were rinsed with an alcoholic solution of thymol, and a few crystals of thymol were placed in each bottle, immediately before each day's collection. At the end of 24 hours, the rats were removed from the cages, and cages and funnels were carefully washed down with boiling water, the washings being collected in the proper bottle. The feces were removed from the cages before this washing, and any contamination of the urine with feces was avoided as much as possible by placing in each funnel a small wad of glass wool, which was rinsed with boiling water in the same manner as the funnels and cages, after the removal of adhering feces.

The data of this experiment are contained in Table VIII.

From Aug. 25 to 28, inclusive, and Aug. 30 to Sept. 2, inclusive, the urines were analyzed as 4 day composite samples. On all other days the daily collections were analyzed separately. Each

collection of urine, whether for 1 day or for 4 days, was used for one analysis.

The data as a whole indicate strongly that the rats were consistently excreting in the urine more nitrate than they were ingesting. We believe that the two 4 day periods above mentioned offer the most reliable data on this point, since the urine collections contained more nitrate, and the error in its determination was, therefore, less than was the case with the daily periods.

TABLE VIII.

Experimental Data on the Nitrate Balance of Albino Rats. Nitrate Values Expressed as Mg. of KNO_3.

Date.	Rat 1 ♂—133.9 gm.			Rat 2 ♀—127.6 gm.			Rat 3 ♀—125.1 gm.			Rat 4 ♂—176.7 gm.		
	Food eaten.	Nitrate intake.	Nitrate in urine.	Food eaten.	Nitrate intake.	Nitrate in urine.	Food eaten.	Nitrate intake.	Nitrate in urine.	Food eaten.	Nitrate intake.	Nitrate in urine.
1915	*gm.*			*gm.*			*gm.*			*gm.*		
Aug. 24	5.6	0.093	1.31	2.9	0.048	1.27	4.9	0.081	0.52	6.7	0.111	0.11
" 25	4.7			4.4			5.5			6.3		
" 26	5.2	0.388	9.77	5.5	0.370	3.74	5.4	0.392	3.00	7.3	0.468	2.68
" 27	5.5			6.3			5.8			9.1		
" 28	8.0			6.1			6.9			5.5		
" 29	5.3	0.088	——*	5.0	0.083	——*	6.4	0.106	——*	8.3	0.138	——*
" 30	5.9			6.8			6.3			7.4		
" 31	4.9	0.364	3.45	5.5	0.364	1.50	6.7	0.403	0.80	5.9	0.476	0.88
Sept. 1	5.2			4.8			5.8			7.0		
" 2	5.9			4.8			5.5			8.4		
" 3	2.9	60.55†	32.36	3.1	80.95†	Lost.	3.2	153.45†	45.50	1.8	61.53†	Lost.
" 4	4.4	0.073	4.01	5.5	0.091	7.86	5.0	0.083	22.35	5.2	0.086	10.02
" 5	5.3	0.088	0.90	4.3	0.071	0.04	6.5	0.108	0.43	6.3	0.105	0.23
" 6	6.5	0.108	——‡	5.7	0.095	——‡	6.1	0.101	——‡	5.9	0.098	——‡
" 7	4.0	43.07†	26.12	3.8	80.36†	53.28	3.9	40.81†	25.43	2.6	48.04†	23.79
" 8	5.0	0.083	3.28	6.0	0.100	4.97	6.1	0.101	4.28	4.5	0.075	1.33
" 9	5.9	0.098	1.71	5.8	0.096	0.20	6.8	0.113	0.46	5.4	0.090	0.27
" 10	4.0	0.066	1.53	5.6	0.093	0.13	5.8	0.096	0.19	3.1	0.051	0.22

* All urines of Aug. 29 were submitted to a qualitative test for nitrate; the diphenylamine test was used. The results were all positive.

† KNO_3 added to food.

‡ All urines of Sept. 6 were also examined qualitatively for nitrates. No clearly positive results were obtained.

On Sept. 3 and again on Sept. 7, varying amounts of KNO_3 were added to the rations of the rats, and in all cases marked increases in the excretion of nitrate in the urine resulted, which persisted over 2 days. The percentage recovery of nitrate in the urine varied from about 45 to 65.

Experiments on Pigs.

Four pure-bred Chester-White pigs were the subjects of the final experiment on the nitrates in the urine.[47] For over 6 months these pigs had been on a feeding experiment and had been in continuous confinement in metabolism stalls. Two were being fed corn-meal and pasteurized skim milk, and the other two rolled wheat and pasteurized skim milk. All water drunk throughout the entire experiment was distilled. Three of the pigs were not in good condition and had been consistently losing in weight for 5 weeks previous to the beginning of the nitrate experiment. About 2 weeks after the close of this test, they were killed and a postmortem examination was made of the carcasses. Several of the internal organs were in an atrophied condition. The fourth pig was in good condition, considering his long confinement, and had gained in weight continuously from the beginning of the experiment.

The nitrate balance of the pigs was determined during the 4 days from Dec. 29, 1915, to Jan. 1, 1916, inclusive. On Jan. 1, the three abnormal pigs weighed 195.0, 173.5, and 190.5 pounds; the normal pig weighed 271.0 pounds.

The three feeds used in this experiment were thoroughly sampled and analyzed for nitrate. The results of the analysis need some explanation. The corn and wheat were analyzed in four 50 gm. samples each, the milk in four 200 gm. samples. The gas obtained from one sample out of each set of four was mixed with a small quantity of air and shaken with a potassium iodide starch solution. The presence of nitric oxide in the gas obtained would lead to the formation of nitrous acid under the conditions of the test, which would liberate the iodine from the KI with the formation of the intense blue color of starch iodide. According to Richardson,[48] this is a very sensitive test. The tests on the corn and wheat were entirely negative, no coloration at all being produced. The test on the milk gave a very faint coloration. A second test which was run on the skim milk was

[47] We wish to thank Mr. J. C. Ross for putting at our disposal the data given on the condition, weight, postmortem examination, and the feed consumption of the pigs used in the following experiment.

[48] Richardson, W. D., *J. Am. Chem. Soc.*, 1907, xxix, 1759.

entirely negative. Water extracts of portions of the corn and wheat also gave no coloration with the diphenylamine test for nitrates; the same result was obtained when this test was run on the filtrate obtained from a sample of the milk after the removal of the proteins by trichloroacetic acid.

The volumes of gas obtained in the course of the regular nitrate method, when shaken with the alkaline permanganate solution, showed decreases, which when figured as nitric oxide gave the following percentages of KNO_3: corn, 0.00496; wheat, 0.0157; and milk, 0.00378. These percentages are comparatively large, but, in view of the negative character of the qualitative tests above mentioned and of the marked tendency of starchy foods to produce other gases than NO absorbable by an alkaline permanganate solution on treatment with ferrous chloride and hydrochloric acid, we believe they are very deceptive. In Table IX are included the food intake, the nitrate intake as calculated from the percentages just given, the volume of urine, and the nitrate in the urine for the four pigs during the 4 days of observation. The food intake of the immediately preceding 5 days is also given.

The following urine collections were submitted to the KI starch test as described above:

Dec.	29	Urine	collections	of	Pigs 315 and 815.
"	30	"	"	"	215 and 815.
Jan.	1	"	"	"	215, 315, and 415.

All of these tests were distinctly positive. No negative tests were obtained on the urines of this experiment.

The data of Table IX afford good evidence that the values given in the table for the nitrate intake, calculated from the volume of gas absorbed by the alkaline permanganate solution, are much too high. This evidence consists of the fact that the much larger feed intake of Pig 815 was not accompanied by anything approaching a correspondingly large nitrate excretion. Everything considered, it seems safe to assume that the feeds of this experiment were nitrate-free, with the possible exception of the milk, but that in the urine small quantities of nitrate were excreted.

The relatively small volumes of urine secreted by the three abnormal pigs, especially by Pig 315, are remarkable. This, of course, necessitated taking smaller samples of urine for analysis. The urine of Pig 815 was analyzed in 400 cc. samples.

TABLE IX.

*Nitrate Balance of Pigs.**

Nitrate Values Expressed as Mg. of KNO_3.

Date.	Pig 215. Weight on Jan. 1, 195.0 lbs.					Pig 315. Weight on Jan. 1, 173.5 lbs.				
	Corn intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.	Wheat intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.
1915	*lbs.*	*lbs.*	*mg.*	*cc.*	*mg.*	*lbs.*	*lbs.*	*mg.*	*cc.*	*mg.*
Dec. 24....	1.0	2.0				1.0	2.0			
" 25....	1.5	3.0				0.5	1.0			
" 26....	1.0	2.0				0.5	1.0			
" 27....	1.0	2.0				1.0	2.0			
" 28....	1.2	2.4				1.2	2.4			
" 29....	1.0	2.0	56.9	264	7.1	1.0	2.0	105.7	602	21.6
" 30....	1.0	2.0	56.9	538	32.0	1.0	2.0	105.7	147	7.0
" 31....	0.5	1.0	28.4	408	8.8	0.5	1.0	52.8	0	0.0
Jan. 1....	1.0	2.0	56.9	335	11.6	1.0	2.0	105.7	375	15.0

Date.	Pig 415. Weight on Jan. 1, 190.5 lbs.					Pig 815. Weight on Jan. 1, 271.0 lbs.				
	Wheat intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.	Corn intake.	Milk intake.	Nitrate intake.	Volume of urine.	Nitrate in urine.
1915	*lbs.*	*lbs.*	*mg.*	*cc.*	*mg.*	*lbs.*	*lbs.*	*mg.*	*cc.*	*mg.*
Dec. 24....	1.0	2.0				4.5	9.0			
" 25....	0.5	1.0				4.5	9.0			
" 26....	0.5	1.0				4.5	9.0			
" 27....	0.5	1.0				4.5	9.0			
" 28....	0.5	1.0				4.5	9.0			
" 29....	1.0	2.0	105.7	745	21.3	4.5	9.0	742.9	3,005	51.1
" 30....	0.0	0.0	0.0	418	19.1	4.5	9.0	742.9	2,318	33.4
" 31....	0.5	1.0	52.8	524	16.9	4.5	9.0	742.9	3,315	23.0
Jan. 1....	1.0	2.0	105.7	622	17.9	4.5	9.0	742.9	2,490	14.0

* The nitrate intake values were calculated from the percentages of KNO_3 in the feeds on the assumption that all of the gas collected and absorbable by the alkaline permanganate solution was NO. According to qualitative color tests, however, the feeds were nitrate-free, with the possible exception of the milk.

CONCLUSIONS.

The experiments above described indicate, in most cases beyond reasonable doubt, that the animal body may excrete in the urine more nitrate than is ingested in the food. The marked differences between the determined nitrate intake and nitrate excretion cannot be accounted for by differences in the accuracy of the method as applied to foods and to urine. The evidence indicates that the urinary nitrates may be determined by our method in a fairly satisfactory manner, since pure KNO_3 added to urines of determined nitrate content could be recovered on an average to the extent of 92 to 94 per cent. The nitrate content of foods cannot be determined in such a satisfactory manner. Nitrates added to foods of determined nitrate content can only be recovered to the extent of 75 to 85 per cent, probably due to the reducing action of sugars. However, this deficit as compared with the equivalent values obtained on urines is probably more than compensated for by the production of gases other than nitric oxide which are retained by the absorbent used, upon treatment with ferrous chloride and hydrochloric acid. This fact is clearly indicated by the analyses in the experiments on pigs.

It seems reasonable to believe, therefore, that the values given for the intake of nitrate in our experiments are high, rather than low, and since there is no reason to doubt the accuracy of the values for the nitrate content of the urine except that they are slightly low, the evidence for the conclusion that the nitrate of the urine is not derived entirely from the food in the case of the subjects experimented upon seems convincing. This conclusion is also supported by the fact, which our own data confirm, that ingested nitrates are destroyed in the body to the extent of 40 to 60 per cent.

There are two possible explanations of the source of the excess of nitrate appearing in the urine over the nitrate ingested with the food. It is conceivable that the nitric and nitrous acids in the air may gain access to the blood in the lungs and ultimately find their way to the urine. Some investigations by Lassar,[49] confirmed by Spiegel,[50] would indicate, however, that nitric acid

[49] Lassar, O., *Z. physiol. Chem.*, 1877–78, i, 165.

[50] Spiegel, A., Ueber die Ausscheidung der Salpetersäure, Inaug. Dissertation, Würzburg, 1894.

fumes are irrespirable. Thus, Lassar showed that if dogs and rabbits on a nitrate-free diet were exposed to concentrated nitric acid fumes, the urine was nitrate-free. Furthermore, assuming a perfect permeability of the lungs to nitric acid, the quantity in which it occurs in the air seems entirely too small to account for the discrepancies observed between the nitrate ingested with the food in our experiments and the nitrate eliminated in the urine. Thus, taking Mabery's[51] figures obtained from analyses of samples of air taken at Cleveland, it may be shown that the total quantity of nitric acid that would be introduced into the lungs per day, on the assumption that the tidal air is 500 cc. and the respiration rate 20 per minute, would average about 5 mg., the maximum being 15.3 mg. The quantity of nitrous acid inhaled is entirely negligible.

The other possible explanation is that the body tissues are able to produce nitrates from unoxidized nitrogenous radicles. It would be mere speculation to attempt a theory of the manner in which this oxidation is accomplished, the intermediate products formed, the particular organ responsible for it if its production is at all localized, or of the biological significance of the process. Such speculations would be profitless in the present state of our knowledge on this question. We hope to investigate the matter further along these lines. We believe, however, that the production of nitrates by animal tissues is a fact and is unique in being the only well established instance of the oxidation of nitrogen, aside from the conversion of nitrites to nitrates, which is apparently a reversible reaction.

[51] Mabery, C. F., *J. Am. Chem. Soc.*, 1895, xvii, 105.

THE DIETARY FACTORS OPERATING IN THE PRODUCTION OF POLYNEURITIS.*

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Eykman,[1] Suzuki,[2] Funk,[3] and others have made it clear that continued feeding of a diet of polished rice induces polyneuritis in birds. Funk[4] has likewise shown that identical results come from feeding birds diets of highly purified foodstuffs. This pathological condition, he has shown, is relieved by the introduction into the diet of water or alcoholic extracts of several natural foodstuffs.

The studies of a number of investigators have now fully established the fact that animals cannot grow when limited to rations of carefully purified proteins, carbohydrates, fats, and salts.[5] McCollum and Davis[6] have shown that certain fats, as butter fat, egg yolk fat, kidney fat, and others contain something which greatly stimulates growth when added to a diet of casein, dextrin, lactose, and a salt mixture of appropriate composition. This substance is present in certain products of plant origin as well. Later they discovered that if the lactose was replaced by dextrin

* Published by permission of the Director of the Agricultural Experiment Station.

[1] Eykman, C., *Arch. path. Anat.*, 1897, cxlviii, 523; cxlix, 187; *Arch. Hyg.*, 1906, lviii, 150.

[2] Suzuki, U., Shimamura, T., and Odake, S., *Biochem. Z.*, 1912, xliii, 89.

[3] Funk, C., *Ergebn. Physiol.*, 1913, xiii, 125, gives an extensive résumé of the literature relating to the so called deficiency diseases, together with a complete bibliography of the older literature.

[4] Funk, *Z. physiol. Chem.*, 1914, lxxxix, 374.

[5] Hopkins, F. G., *J. Physiol.*, 1912, xliv, 425. Hopkins, F. G., and Neville, A., *Biochem. J.*, 1913, vii, 97. Funk, C., and Macallum, A. B., *Z. physiol. Chem.*, 1914, xcii, 13. Stepp, W., *Z. Biol.*, 1913, lxii, 405.

[6] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxi, 179.

in this ration the addition of butter fat did not induce growth. When this diet, containing butter fat, was supplemented with small additions of the water extract of boiled egg yolk, or water or alcoholic extracts of wheat germ, growth proceeded at the normal rate. They formulated the working hypothesis that in addition to the recognized essential constituents of a successful diet, *viz.*, protein, carbohydrate, fats, and inorganic salts, the growing animal requires two substances, or groups of substances; one, which is soluble in fats, is contained in fats from certain sources; the other, which is soluble in water and alcohol, is found widely distributed both in the animal and vegetable worlds.

Funk and Macallum[7] have pointed out that butter fat does not relieve polyneuritis in pigeons and also that with rations consisting, aside from the butter fat content, of carefully purified foodstuffs, does not induce growth in young rats and they clearly express their belief that "vitamines" are absent from butter fat.

By "vitamine" Funk and his coworkers indicate a class of substances the chemical nature of which is at present unknown. They are described as being precipitated by phosphotungstic acid and by mercuric chloride, but aside from this property there is no evidence that these substances of unknown constitution which are active in promoting growth, or in relieving the symptoms of polyneuritis contain an amino group. Furthermore, the prefix *vita* connotes an importance in biological processes paramount to that of certain other absolutely indispensable organic complexes, among which are a number of amino-acids. We feel that this term is not in harmony with a conservative tendency in the nomenclature of biological chemistry which should avoid the employment of a term which carries the idea that one indispensable complex is of greater importance biologically than another one equally indispensable. Furthermore, the evidence of the presence of an amino group in the substances under consideration is too slight to warrant the use of the ending *amine*, which carries with it a definite meaning in organic chemical nomenclature.

Hopkins[8] introduced the term "accessory" for the substances of unknown chemical character which are found in milk and which

[7] Funk and Macallum, *J. Biol. Chem.*, 1915, xxiii, 413.

[8] Hopkins, *J. Physiol.*, 1912, xliv, 425. Hopkins and Neville, *Biochem. J.*, 1913, vii, 97.

render the addition of small amounts of milk to rations consisting otherwise of purified food substances, so effective in promoting growth. The term accessory carries with it the idea of playing a subordinate rôle, and in this sense has been employed by writers on dietetics as synonymous with condiment.[9] While we have, following Hopkins, employed this term up to the present time, we have felt that it is by no means satisfactory.

We would, therefore, suggest the desirability of discontinuing the use of the term vitamine, and the substitution of the term fat-soluble A and water-soluble B for the two classes of unknown substances concerned in inducing growth. These terms have the merit of not attributing extravagant values to these bodies, and they differentiate between the substances or groups of substances only with respect to their solubility relations, which is the only basis of differentiation at present known. These terms have the additional advantage that further letters may readily be introduced as investigation progresses, provided there proves to be more than a single representative of each class, and also that they will automatically fall into disuse when we come to possess definite knowledge of their chemical nature.

When McCollum and Davis[10] proposed the working hypothesis regarding the essential factors in the diet during growth which assumes the necessity of the two kinds of unknown substances mentioned above, they were fully aware of the apparent discrepancy between this hypothesis and the recorded experimental data which indicated that birds can be cured of polyneuritis by the employment of water extracts of certain natural foodstuffs. Such water extracts could scarcely be assumed to contain appreciable amounts of lipoidal material. In fact, nearly three years ago, one of us (M.) satisfied himself by experimental trial of the validity of the statement that a cure can be effected in this way.

In the present paper we wish to report an investigation of the relation to polyneuritis of the two classes of unknown dietary factors A and B as defined above.

[9] Howell, W. H., Textbook of Physiology, Philadelphia, 5th edition, 1913, 727, 905.

[10] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 231.

Effects of Feeding Polished Rice with Butter Fat.

Bird 792A was offered a ration of rice 95 and butter fat 5 per cent from Sept. 18 to 28. It steadily lost weight and was evidently not consuming an adequate amount of food. Beginning with Sept. 28 we forcibly fed the pigeon with 20 gm. daily of the rice and butter fat mixture. On Oct. 15 it manifested the first symptoms of polyneuritis. Oct. 16, it was in a very bad condition. It was given the alcoholic extract (95 per cent) of 10 gm. of wheat embryo. Oct. 17, the bird was in a nearly normal condition.

In all, nine birds were fed with this ration and the above protocol illustrates what may be anticipated as the result of confinement to this diet.

Our observations are in accord with those of Funk and Macallum indicating that butter fat does not protect against polyneuritis birds fed polished rice and the fat.

We early became convinced that pigeons cannot be depended upon to eat enough of such experimental rations as are described in this paper, so we adopted the practice of forced feeding twice daily. This was accomplished by inserting a tube into the crop and pouring the powdered ration into it. A glass rod served to discharge the food from the tube when necessary. About 10 gm. of food were administered at each feeding. Water was given twice daily in 10 cc. doses by means of a pipette.

Effects of Feeding Polished Rice and Alcoholic Extracts of Fat-Free Wheat Embryo.

500 gm. of wheat embryo were freed from fat by ether in a continuous extractor and the residue was then extracted during 18 hours with 95 per cent alcohol also in a continuous extractor of the Caldwell type. The alcoholic extract was evaporated on 200 gm. of dextrin (Preparation 1).

Bird 1113B was offered rice for voluntary consumption from Nov. 11 to 18. After this date the bird was forcibly fed with a mixture of casein 18, salts 3.7, dextrin 78.3 per cent.

This ration will be hereafter referred to as the casein ration. The composition of the salt mixture employed was as follows:

	gm.		gm.
NaCl	0.173	$CaH_4(PO_4)_2 . H_2O$	0.540
$MgSO_4$ (anhydrous)	0.266	Ca lactate, $Ca(C_3H_5O_3)_2 . 5H_2O$	1.300
$NaH_2(PO_4) . H_2O$	0.347	Fe lactate Merck	0.118
K_2HPO_4	0.954		

Funk[4] employed a ration of similar composition but containing butter fat, and observed that the birds were stricken wth polyneuritis after a period comparable to rice feeding.

Nov. 26, the pigeon was in the typical condition of severe polyneuritis. It was given Preparation 1 equivalent to 10 gm. of wheat embryo, morning and evening. Nov. 27, it was able to sit in a normal position, but could not use its legs. It was fed as on the 26th. Nov. 28, it was given 10 gm. of Preparation 1 and 10 gm. of the casein ration. Nov. 29, the bird could walk. We continued feeding as on Nov. 28 to Jan. 12 when the experiment was discontinued, the bird being in a normal condition.

The presence of the fat-soluble A in wheat embryo was established by McCollum and Davis.[6] They did not study the growth-promoting power of the isolated wheat embryo fat, however, and only after these experiments were well under way did we learn in another connection in feeding work with rats that the fat may be quantitatively removed from wheat embryo and yet the fat-free residue, when added to a ration of purified food materials, can induce in rats a growth curve entirely unlike those obtained with diets lacking the unknown A.[11] This led us to conclude that the recovery from polyneuritis and prolonged well-being of pigeons given alcoholic extract of fat-free wheat embryo, was not a demonstration of recovery and subsequent maintenance on a diet free from the fat-soluble A, since this is in the fat-free embryo and might be extracted with alcohol. We next sought to induce the recovery of pigeons by the administration of nearly lipoid-free preparations from other sources.

Experiments with Potato Juice.

Vedder and Clark[12] employed potato juice and found it inactive. Our experiments with potato juice gave results indicating that it possesses a moderate curative power.

Bird 1169B was forcibly fed from Nov. 14 to Dec. 4 with rice until polyneuritis was imminent. It was continued on rice and from Dec. 4 was given 12 cc. of fresh potato juice twice daily. The bird could walk very unsteadily from Dec. 8 to 18 when it so improved as to walk naturally, flew voluntarily to a perch, and appeared normal. The potato juice

[11] In a subsequent paper from this laboratory soon to appear this point will receive full consideration.

[12] Vedder, E. B., and Clark, E., *Philippine J. Sc., B.*, 1912, vii, 423.

was continued until Jan. 12 when the forced feeding was discontinued, the bird appearing normal. The ration of rice with potato juice evaporated upon it was offered for voluntary consumption. The consumption was entirely inadequate and the bird rapidly lost weight and strength during the first week. On changing it to a diet of corn meal it regained its normal condition.

Since the pigeons invariably grow emaciated when given diets which induce polyneuritis and may fully recover with proper dietary treatment without appreciable increase in weight, together with the fact that considerable fluctuation in body weight may result from the retention of food in the crop as the time for the onset of the pathological symptoms approaches, we do not believe that the value of our records is enhanced by the inclusion of the weight records. This portion of our data is therefore omitted.

Experiments with Cabbage Juice.

Bird 862B. This bird was forcibly fed with 25 gm. of polished rice per day from Nov. 11 to Dec. 6. From Nov. 24 the crop was continually packed with food and very hard. Locomotion was distinctly impaired. On Dec. 6 and thereafter two doses of cabbage juice of 15 cc. each were administered daily—morning and evening. The crop became soft when rice and cabbage juice were both administered and on Dec. 8 the bird walked much better. It never returned to a normal condition, however, and although the regular feeding with this mixture was continued, it occasionally regurgitated food and on Dec. 19 the crop was again packed with food. Dec. 20, it was in the typical polyneuritic condition. Three doses of cabbage juice were given on this date and the bird was still alive on the morning of the 21st and very weak, but the head was not retracted. Dec. 21, five doses of cabbage juice representing a total of 180 cc. evaporated to a pasty consistency were given. The symptoms of polyneuritis were in great measure relieved, but the bird was very weak and died at 9 a.m., Dec. 22. There is no doubt that the cabbage juice contains the curative factor, but apparently in a quite low concentration.

Bird 1113 B. Forcibly fed with rice from Jan. 12 to 29 when it showed by its retracted head and inability to walk that it was in the polyneuritic state. In the morning we gave it cabbage juice (15 cc.) and 10 gm. dextrin on which 70 cc. of cabbage juice had been evaporated. In the afternoon the same dosage was administered. Jan. 30, the bird was normal in its movements and appeared cured.

Experiments with Oat Extract.

Bird 1091H was forcibly fed polished rice from Nov. 12 to Dec. 6 when symptoms of polyneuritis appeared. It was fed Dec. 6, morning and

afternoon, 12 gm. of rice on which a water extract of 24 gm. of rolled oats had been evaporated. 500 gm. of finely ground rolled oats were shaken in a shaking machine with 2 liters of water for 1 hour. The material was then centrifuged and the supernatant liquid poured off and evaporated on 500 gm. of rice. At each feeding 12 cc. of water were introduced into the crop. This treatment was thereafter continued daily until Dec. 16. The recovery was slow, but progressive, the crop remaining hard. On Dec. 12 the crop was washed out with a stream of warm water introduced through a rubber tube. The bird was apparently normal on Dec. 16 and thereafter would fly up to a perch and remain there when not disturbed. Forced feeding of this ration was continued until Jan. 19. For 3 weeks preceding the termination of the experiment this bird was very spirited and would persistently attack and annoy any other bird which was introduced into its cage, so it had to be kept isolated.

This experiment is of special interest because of the behavior of rats when fed rolled oats alone as contrasted with a mixture of rolled oats plus butter fat. When confined to rolled oats or the oat kernel freed from hulls in the laboratory, young rats steadily lose weight. On adding butter fat (5 per cent) to the oats they slowly gain in body weight and continue to exhibit a well nourished appearance for months.[13] From this we draw the conclusion that the oat kernel contains little if any of the fat-soluble A, since without its addition even maintenance is not attained, while growth proceeds when this factor is added. Since a solvent such as water, which exerts a very slight solvent action on lipoidal material, removes from oats a substance which cures polyneuritis, it points strongly to the belief that the cure is affected without the intervention of the fat-soluble A. The possibility remains, of course, that the requirement of the fat-soluble A is small for maintenance as compared with the amount necessary for growth. Since both classes of these unknown essential components of the diet are present in the vegetable world, and we have as yet no method perfected for testing for very small quantities of either A or B, this point cannot yet be definitely decided. Since, however, a pigeon can be maintained in excellent condition, as was Bird 1091H, by a mixture of 20 gm. of rice plus the water extract of 24 gm. of rolled oats per day, and growth cannot be attained with the content of the fat-soluble A in 100 per cent of rolled oats, we feel safe in asserting that the maintenance requirement of the

[13] An extended study of the dietary deficiencies of the oat kernel is now in progress in this laboratory and will be reported in a later paper.

bird for the fat-soluble A is less than the growth requirement of the rat.

There appears to be good evidence that the fat-soluble A tends strongly to remain associated with non-lipoid materials. This we have mentioned above in connection with the growth-promoting power, with respect to this particular factor, which is exhibited by wheat embryo completely extracted with ether. Further support of this idea is found in the growth curves obtained by Osborne and Mendel[14] with their protein-free milk. These curves showing normal growth over periods of 3 months or more could have been obtained only with rations which provide this dietary factor. Since they have shown that ether extracts almost nothing from protein-free milk it follows that it is difficult to free food materials from this substance.

We have endeavored to advance in the matter of determining whether water extracts of lipoid-free foodstuffs can effect a cure of polyneuritis in pigeons. To this end 500 gm. of wheat embryo were extracted exhaustively with ether and afterwards with carbon tetrachloride. The extraction with carbon tetrachloride for $22\frac{1}{2}$ hours at a good rate in a continuous extractor removed only 0.07 per cent of material from the ether-extracted embryo. The residue after exhaustion with both these solvents was extracted with water by stirring the embryo into boiling water and adding acetic acid to facilitate the complete coagulation of dissolved protein. The material was centrifuged and the clear supernatant liquid was evaporated on 500 gm. of dextrin (Preparation 2). This product had curative properties in a high degree.

Bird 910A. Fed rice Dec. 21 to Jan. 10 when it manifested typical polyneuritis. Jan. 10, it was given two doses of 5 gm. each of the casein ration and of Preparation 2. Jan. 11, p.m., the bird was slightly improved, but was in danger of dying during the night, so it was given a dose of the curative substance prepared by extracting 10 gm. of Preparation 2 with water. Jan. 12, a.m., the bird was in a normal condition. Thereafter it was given the casein ration carrying the Preparation 2 equivalent to 5 per cent of lipoid-free germ. On Jan. 16 its crop was hard and only water was given in the morning. In the evening the crop was still packed and hard and the bird was again showing incipient polyneuritis so it was given the strong solution from 10 gm. of Preparation 2. Jan. 17, a.m., the bird was again

[14] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1912, xii, 81.

normal. Thereafter it was given daily 20 gm. of the casein ration carrying Preparation 2 equivalent to 10 per cent of lipoid-free embryo. It remained normal until Jan. 23, p.m., when it again had polyneuritis. It recovered again with the strong solution, and the experiment was discontinued.

This record was duplicated in all essentials with two other pigeons, and leaves no room to doubt that the water extract of wheat germ made lipoid-free by thorough extraction with ether and with carbon tetrachloride induces a complete recovery from polyneuritis.

Experiments with Acetone Extract of Fat-Free Wheat Embryo.

For the isolation of the curative agent it is of the utmost importance to have an extensive knowledge of the solubility of the substance in all the ordinary solvents. With a view to obtaining such evidence we have administered to polyneuritic pigeons the extract of wheat embryo prepared by first removing all ether-soluble matter, and then extracting the residue at a vigorous rate in an extractor of the Caldwell type with acetone for 18 hours. The acetone-soluble material from 500 gm. of wheat embryo, which amounted to 3.2 gm., was incorporated with 396.8 gm. of dextrin (Preparation 3).

Bird 1096. Ate polished rice voluntarily from Dec. 14 to Jan. 13, when it was in incipient polyneuritis. It was given water extract of 10 gm. of Preparation 3. Jan. 14 signs of paralysis had disappeared. Gave the same water extract and rice in the morning. In the afternoon gave water only, as the crop was still hard. Jan. 15, the bird was in good condition. Fed the casein ration carrying the acetone extract of 5 per cent of wheat germ. Jan. 18, the crop was hard so we gave water only. Jan. 19, the crop was empty and the usual food given. Jan. 20, the crop was rather hard, the bird walked unsteadily. It was given 10 gm. of food in the morning; only water in the afternoon as the crop was packed. Jan. 21, the crop was hard, the bird walked unsteadily. It was given the casein ration carrying 10 per cent of acetone extract of embryo, but otherwise like that fed after Jan. 15. Jan. 22 the bird had a severe attack of polyneuritis in the morning. We gave strong water extract of 10 gm. of Preparation 3. Repeated this at noon. In the afternoon it was still very ill, so it was given another dose at 4 o'clock. At 8 p.m. it was on its feet. The dose was repeated. Jan. 23, it was nearly normal. We gave it the water extract of 10 gm. of Preparation 3 plus 5 gm. casein ration. Jan. 24, the bird was in normal condition, and the experiment was discontinued.

The above protocol is typical of three records obtained with acetone extract of wheat embryo. The curative substance is extracted by this solvent, in the course of long continued extraction.

Experiments with Benzene Extract of Fat-Free Wheat Embryo.

1 kg. of wheat embryo was extracted for 18 hours with ether and the residue afterward extracted in a Caldwell extractor for 18 hours with benzene. The benzene was evaporated on 200 gm. of dextrin. The extract amounted to 3.6 gm. (Preparation 4).

Bird 1172 from Dec. 14 was fed the casein ration. On Jan. 19 it began to regurgitate its food. Jan. 23, it had a bad attack of polyneuritis and was given a water extract of 10 gm. of Preparation 4, morning and evening. Jan. 24, it was much better, but still had symptoms of the disease. The same treatment was repeated. Jan. 25, it was nearly normal. Gave it 10 gm. of Preparation 4 plus 10 gm. of the casein ration in the morning. In the afternoon it appeared normal.

Another bird was cured after a similar history by this preparation.

Experiment with Ethyl Acetate Extract of Fat-Free Wheat Embryo.

400 gm. of wheat germ were extracted with ether during 48 hours. The residue was extracted for 14 hours with ethyl acetate and the ethyl acetate evaporated on 400 gm. of dextrin (Preparation 5).

Bird 968B was forcibly fed with rice from Dec. 14 to Jan. 31 when it had a severe attack of polyneuritis. It was given a water extract of Preparation 5, equivalent to 10 gm. of wheat embryo, in the morning. In the afternoon of Jan. 31 it could walk fairly well, and the dose was repeated. Feb. 1, the bird was in a normal condition.

The results of these experiments help in clarifying the apparent lack of harmony in the dietary requirements of the mammal during growth, and the relief of polyneuritis in birds. Both the fat-soluble A and the water-soluble B must be present in the diet of the mammal during growth. The absence of the former leads to failure to grow, to emaciation, and to a pronounced inflammation of the eyes, all of which conditions, provided the other factors in

the ration are adequate,[15] are promptly relieved by the administration of fats of the group which possess the peculiar properties of butter fat. No matter how generous may be the supply of the fat-soluble A, failure of nutrition will result, provided an adequate amount of the water-soluble B is not also furnished by the diet.[16] Judging from the appearance of serious nutritional disturbances ending in death, which result from a shortage of the fat-soluble A, and the emaciation, weakness, and death which follow restriction to a diet inadequate in its content of the water-soluble B, *it seems certain that both these classes of unknown dietary constituents are essential for maintenance as well as for growth.*

SUMMARY OF CONCLUSIONS.

1. The data presented in this paper show conclusively, we believe, that in the production of polyneuritis in birds by exclusive rice feeding or exclusive feeding of a ration made up of purified foodstuffs, the degeneration of the nerve cells is the specific result of a lack of the water-soluble B. *The fat-soluble A appears to be dispensable when maintenance alone is involved, for a somewhat longer period than is the factor B.*

We base this last assertion upon the fact that pigeons can be brought into the polyneuritic state by feeding a diet free from both the essential factors A and B, and can be completely cured and maintained in a normal condition for at least 35 days on the same diet which brought on the disease, plus the water extract of a foodstuff (rolled oats) on which rats cannot grow without the addition of butter fat, but on which they do grow when the latter is added. Confirmatory evidence is also offered in our success in inducing relief from polyneuritis in birds, by treatment with water extract of wheat embryo, previously rendered lipoid-free by exhaustion with ether and carbon tetrachloride successively, and also in our success in curing birds with such substances as cabbage or potato juice, both of which are practically free from lipoids. The results with these lipoid-free products, we present

[15] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 180. Osborne and Mendel, *J. Biol. Chem.*, 1913, xvi, 431.

[16] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181, 199, Chart 5, and 221, Chart 33.

with a full appreciation of the fact that the fat-soluble A is found in considerable amount in such natural foods as wheat germ even after practically complete removal of the lipoids.

2. We also present experimental data showing that acetone, benzene, and ethyl acetate extract from wheat embryo, previously rendered fat-free by extraction with ether, the substance which relieves the symptoms of polyneuritis in pigeons.

A COLORIMETRIC METHOD FOR THE ESTIMATION OF AMINO-ACID α-NITROGEN. II.

APPLICATION TO THE HYDROLYSIS OF PROTEINS BY PANCREATIC ENZYMES.

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In the first communication on this subject[1] the authors showed that the interaction of amino-acids and triketohydrindene hydrate (ninhydrin reaction) in presence of pyridine could be used as a means of estimating amino-acid α-nitrogen colorimetrically. The amount of blue coloring matter (ammonium salt of diketohydrindylidene-diketohydrindamine) formed is proportional to the amount of amino-acid α-nitrogen when the latter is between the limits of 0.05 mg. and 0.005 mg. per cc. of liquid to be estimated. It was claimed that the method could be used as a means of following protein hydrolysis and this present paper contains the evidence in support of such a belief.

We have also subjected to criticism and reexamination the matter of our previous paper where it affects the method for our colorimetric determination, and a year's experience with the process has shown us one or two points which require amplification.

The Standard Alanine Solution.—The standard alanine solution is made as previously directed by dissolving 0.3178 gm. of freshly crystallized alanine in water and making up the volume to 1 liter. Its stability varies a little. Thus one solution was found to be stable for a period of 3 months while a second only gave about 95 per cent of the theoretical amount of standard color at the end of that time. In consequence we have found it advisable to check the stability of our standard alanine by comparing it from time to time with a freshly prepared solution.

The Standard Color.—We have made several further attempts to prepare a permanent standard color, but without success. The

[1] Harding, V. J., and MacLean, R. M., *J. Biol. Chem.*, 1915, xx, 217.

direct preparation of the ammonium salt of diketohydrindylidene-diketohydrindamine by the interaction of a mixture of ammonium acetate and carbonate upon hydrindantin[2] is anything but quantitative, and conditions have not been found under which the blue coloring matter is absolutely stable. We have also attempted to prepare artificial standards from mixtures of various dyestuffs and coloring matters. The only partially successful experiment was a mixture of phenolphthalein and thymolphthalein in alkaline solution. This matched the standard alanine color perfectly, but possessed no advantages over it, as it was even less stable. The standard color is prepared as previously directed. 1 cc. of 10 per cent aqueous solution of pyridine may replace the 0.5 cc. recommended, if it is so wished.

The stability of the standard color seems to vary. Our first statement is undoubtedly incorrect, and we can only ascribe the error to some confusion of notes. The minimum time of stability of the standard color which we have observed is 3 hours; the maximum is 12. Hence we cannot recommend the use of a standard color which has been prepared over a period longer than 3 hours.

The production of the color in the standard, and hence in the determinations, is in all probability a quantitative one in the absolute sense. Thus it cannot be increased by the use of further excess of either pyridine or triketohydrindene hydrate.

1 cc. standard alanine.		Colorimeter reading.
1 cc. pyridine. Concentration.	1 cc. ninhydrin. Concentration.	
per cent	*per cent*	
5	1	1.07
10	1	1.00
10	2	1.01
15	1	0.98
25	1	1.00

The use of 1 cc. of 5 per cent aqueous solution of pyridine, however, gives too low a result. This is interesting as 0.5 cc. of 10 per cent aqueous solution of pyridine gives the maximal value.

[2] Ruhemann, S., *J. Chem. Soc.*, 1911, xcix, 1486.

Maximal and Minimal Amounts of Amino-Acid α-Nitrogen Determinable by the Method.—These two points were fixed at 0.05 mg. and 0.005 mg. nitrogen per cc. Judging by our experience with the various proteins cited in this paper, the method gives accurate results with 0.08 mg. of amino-acid α-nitrogen per 1 cc. When dealing with the mixtures of the amino-acids it appears quite a safe procedure to estimate the α-nitrogen in a higher concentration than is possible with some of them by themselves, and this is, of course, to be expected. Even this value is probably too low, but we prefer to err on the safe side. The lower limit, however, is perhaps too low for practical convenience in this field of work. It is possible to obtain accurate results at that concentration of nitrogen, but they seem to be extremely susceptible to error, and if it is possible, we would not advise the use of a limit lower than 0.01 mg. amino-acid α-nitrogen per cc. Even then great care must be taken in the determination.

The Case of Cystine.—As was pointed out in the previous communication, cystine is the one amino-acid among those examined which fails to give quantitative results. Furthermore we have not been able to devise any modification whereby this amino-acid can be estimated by our colorimetric process. The colors produced are always very red and very low in amount, due to the formation of either excessive amounts of hydrindantin or sulfur derivatives. Judged by the Van Slyke method as standard, however, it does not seem to interfere in the estimation of the amino-acid α-nitrogen in the majority of hydrolyzed proteins, thus bearing out our contention of the previous paper.

Method of Estimation of Amino-Acid α-Nitrogen by the Colorimetric Process.

1 cc. of the solution to be estimated, containing between 0.08 and 0.01 mg. of amino-acid α-nitrogen and neutral to phenolphthalein, is mixed with 1 cc. of 10 per cent aqueous solution of pure pyridine, and 1 cc. of a freshly prepared 2 per cent solution of triketohydrindene hydrate (ninhydrin), in a test-tube, and stoppered lightly with an absorbent cotton tampon. The test-tube is placed in a rapidly boiling constant level water bath for a period of 20 minutes, then removed, cooled by immersion in cold water, and diluted to a suitable volume, which must be arranged so that

the colorimetric readings do not differ greatly from the reading of the standard color. The comparison is made in a Duboscq colorimeter, placed in a dark room, and illuminated as described in our previous paper (page 221), and too much stress cannot be laid on this point.

The standard color is prepared by similarly heating 1 cc. of standard alanine solution, 1 cc. of 10 per cent aqueous pyridine, and 1 cc. of 1 per cent solution of triketohydrindene hydrate, for a period of 20 minutes, cooling, and diluting to 100 cc. In estimating solutions containing small amounts of amino-acid α-nitrogen (0.03–0.01 mg. per cc.), it is advisable to use a standard of half the ordinary strength (1 cc. = 0.025 mg. of nitrogen) and dilute the color to 50 cc. It is important that the water bath used in heating the solutions shall be large and of constant level, otherwise the heating may become uneven and irregular results be obtained.

Application of the Method to the Study of the Hydrolysis of Proteins by Pancreatic Enzymes.

In order to determine experimentally the application of our colorimetric method to a study of proteoclasis, we have followed by means of it the hydrolysis of various types of proteins by pancreatic enzymes, and compared the results with those obtained by the Sörensen and Van Slyke methods. The use of the pancreatic enzymes, as proteoclastic agents, gives results which are not complicated by other factors, such as high acidity or the production of ammonia,[3] and thus makes all determinations simpler and less subject to error. The hydrolysis was allowed to proceed for 3 or 4 days in presence of toluene until the amino-acid α-nitrogen value had become almost constant. It is to be noted, however, that in no case did the values ever become absolutely constant, though the rate of hydrolysis became very small, yet it never entirely ceased; even at the end of 216 hours the hydrolysis of casein was still proceeding.

The proteins used in these experiments were serum albumin and globulin (Schuchardt), gluten (Schuchardt), fibrin (ox blood), gelatin, casein (purified), nucleoprotein (Schuchardt), peptone (Witte),

[3] Andersen, A. C., *Biochem. Z.*, 1915, lxx, 344.

and peptone *e carne* (Schuchardt). They were used in solutions of approximately 0.5 per cent, and to 100 cc. of the protein solution were added 1 cc. of the pancreas preparation, 1 cc. of 0.5 per cent sodium carbonate solution, and 2 cc. of toluene. Several such solutions were made and all incubated together at 38–39°C.

The enzyme solutions used were two in number. The first was a preparation of the pancreas by Fairchild Brothers and Forster, and sold under the name of *holadin*, and was used in either a 0.2 or a 1 per cent solution. This preparation is supposed to contain trypsin. We found, however, that although it was active towards casein, gelatin, and peptones, it was entirely inactive towards serum albumin and globulin, thus corresponding in its properties to erepsin rather than to trypsin. We later found that its proteoclastic powers could be extended to fibrin and to gluten. There is undoubtedly, even yet, much uncertainty and confusion existing about the activities of these two classes of proteoclastic enzymes, and the age of our sample may have been the cause of our result. Nevertheless, as the enzyme was inactive towards albumin and globulin, we prefer to class it as the pancreatic erepsin discovered by Vernon[4] rather than as trypsin. It did not belong to the autolytic class of enzymes producing large quantities of ammonia. We examined this point experimentally and found the following amounts of ammonia produced by the action of this enzyme on a 0.5 per cent solution of protein in 100 hours, and compared them with the amounts of amino-acid α-nitrogen.

100 cc. of the protein solution (0.5 per cent).	Ammonia nitrogen.	Amino-acid α-nitrogen.
	mg.	*mg.*
Casein	0.67	22.7
Peptone (Witte)	0.28	24.3
Peptone *e carne*	0.44	17.0

The small amounts of ammonia are thus insignificant compared with the amounts of amino-acid α-nitrogen.

The second enzyme preparation was an alcoholic extract of pig's pancreas prepared according to the directions given by Cole.[5] This agreed in its proteoclastic properties with trypsin.

[4] Vernon, H. M., *J. Physiol.*, 1904, xxx, 330.

[5] Cole, S. W., Practical Physiological Chemistry, Cambridge, 1913, 3rd edition, 94.

At the end of stated intervals the flasks containing the hydrolysis experiments were removed from the thermostat, boiled for about 15 seconds, and the amino-acid α-nitrogen content was determined by the three aforementioned methods.

The colorimetric method was applied, as stated in this paper, directly to the solution. The alkalinity caused by the presence of the sodium carbonate was found to be too small to affect the results.

In the Van Slyke method we used the micro form of the apparatus.[6] Care was taken in the standardization of the apparatus and the use of reagents.

To apply the Sörensen method 20 cc. of solution neutral to phenolphthalein were taken, excess of neutral formaldehyde was added, and then titrated with 0.1 N NaOH, until a deep pink solution resulted, the color of which was permanent a full 5 minutes.

The general results can best be seen by an inspection of the curves shown in Figs. 1 to 8.

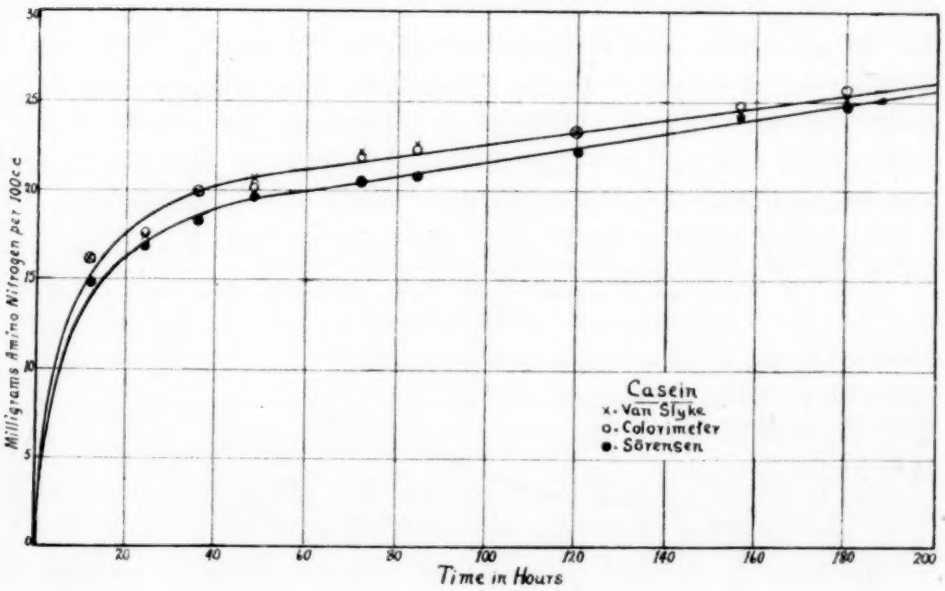

FIG. 1.

[6] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121.

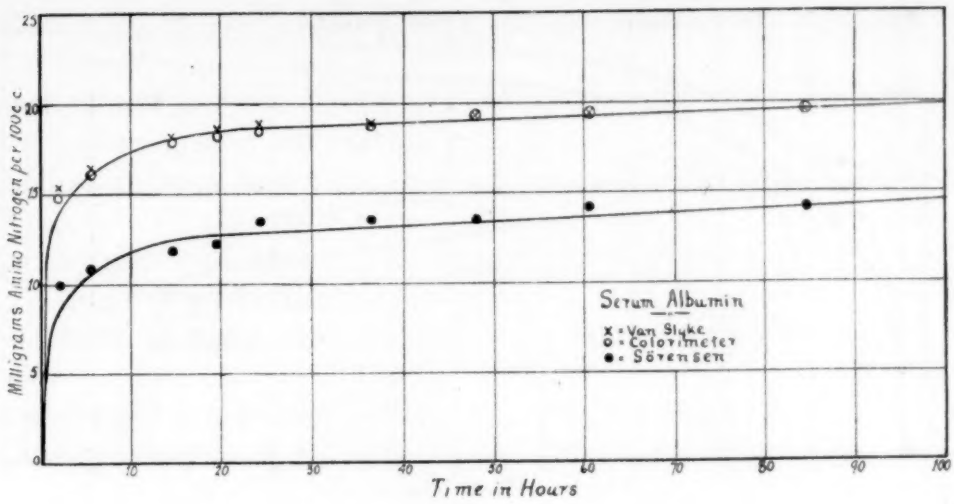

FIG. 2.

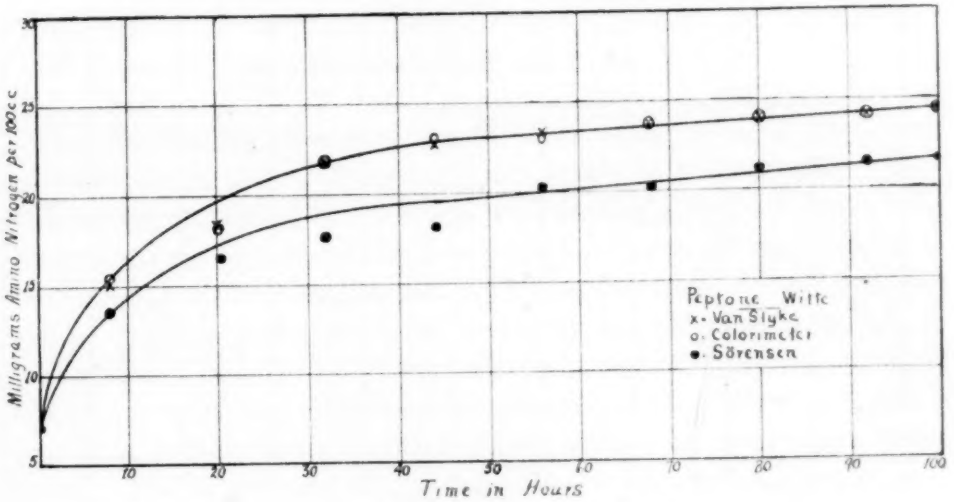

FIG. 3.

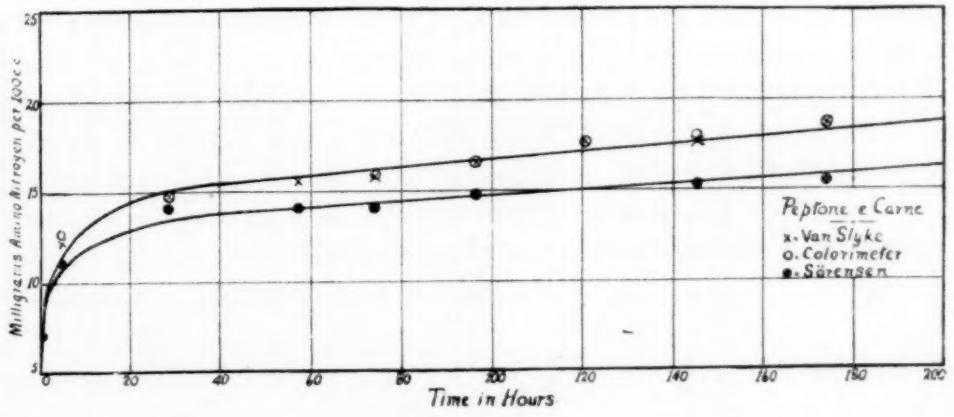

FIG. 4.

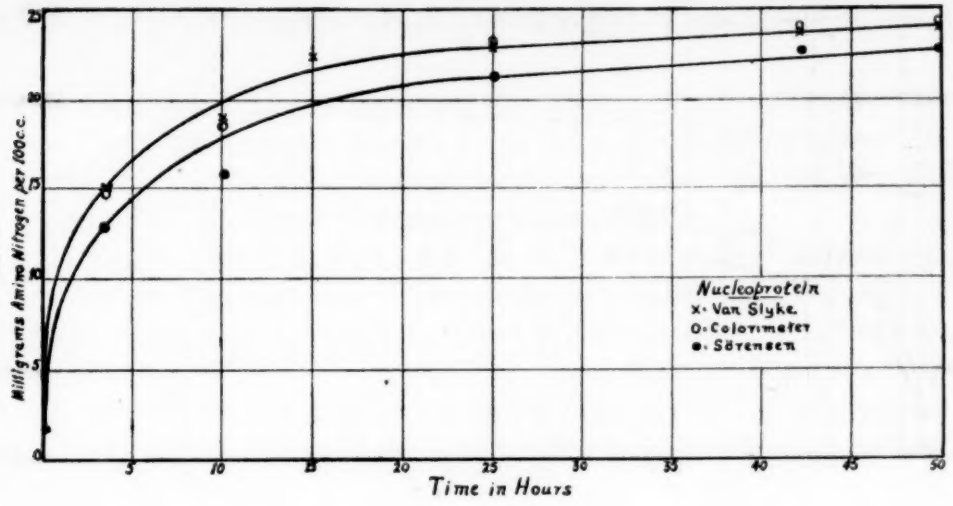

FIG. 5.

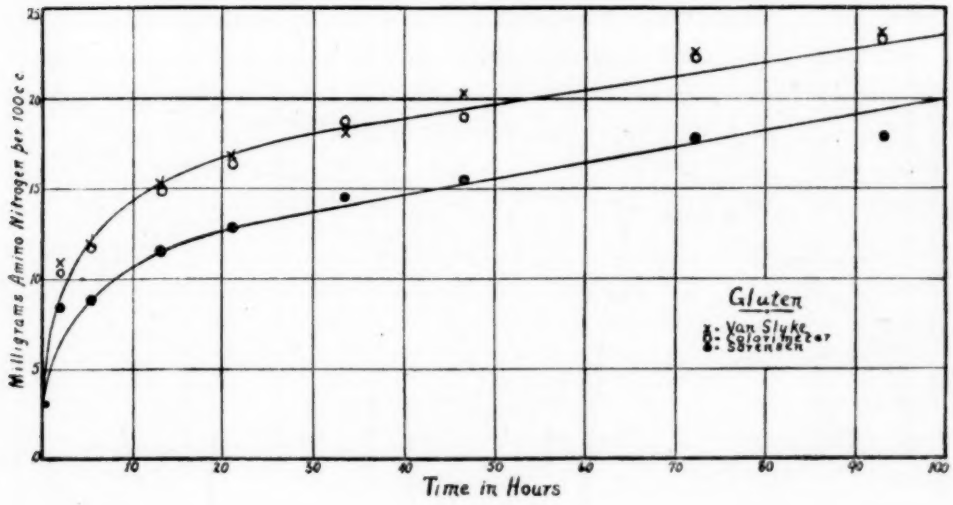

FIG. 6.

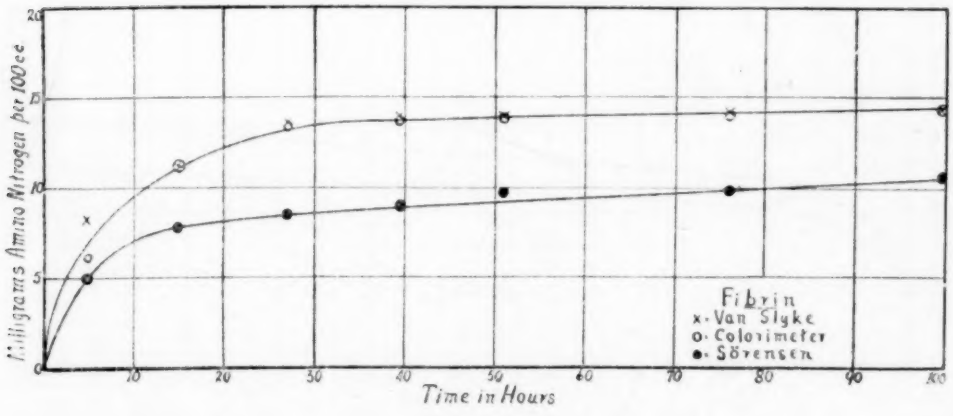

FIG. 7.

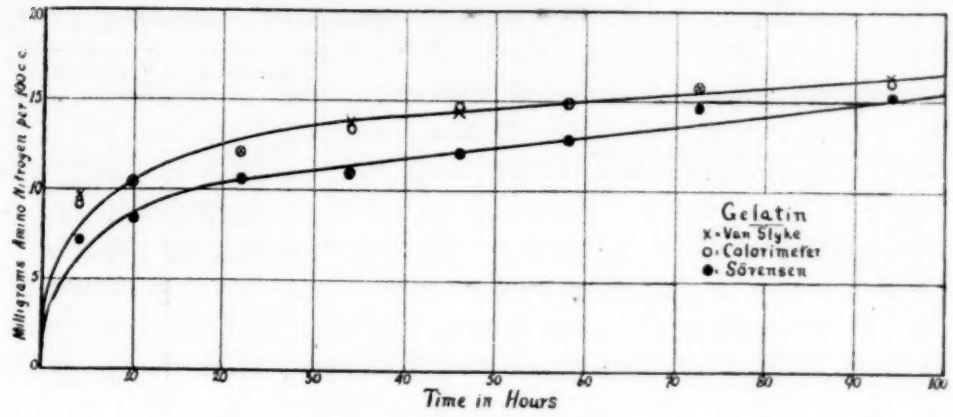

FIG. 8.

DISCUSSION OF RESULTS.

The results of the experiments show quite clearly that the colorimetric method is applicable to a study of proteoclasis. The striking agreement of the method with the Van Slyke method and the general agreement of the two with the Sörensen method show that it estimates quantitatively the hydrolysis of the peptide chain. Qualitatively all three methods give the same information, and can be used to determine the presence of peptide groupings in an unknown substance or mixture, or to detect the presence of a proteoclastic enzyme when acting on a peptide substrate. The agreement of the colorimetric and gasometric methods is to be expected, for both give theoretical results with aqueous solutions of pure amino-acids, except in the case of glycocoll with the gasometric method, and cystine with both methods. Both methods give identical results when applied to peptone solutions obtained from various sources. A reference to the experimental figures, however, will show that with some native proteins and during the early stages of the hydrolysis of these proteins the two methods do not give identical results. This is shown in the following table, very clearly in the case of the mixture of serum albumin and globulin, and of fibrin.

Protein.	Time.	N_2 per cc.	
		Van Slyke.	Colorimeter.
	hrs.	*mg.*	*mg.*
Serum albumin and globulin	0.0	0.0612	0.0200
Gluten	0.0	0.0313	0.0293
Fibrin	5.0	0.0833	0.0602
Gelatin	1.0	0.0352	0.0320
Casein	0.5	0.0386	0.0384
Nucleoprotein	0.0	0.0174	0.0176
Witte peptone	0.0	0.0734	0.0719
Peptone *e carne*	0.0	0.0777	0.0807

As the hydrolysis proceeds, however, the figures begin to come into agreement and at the end of 15 hours they read:

	Van Slyke.	Colorimeter.
	mg.	*mg.*
Serum albumin and globulin	0.1812	0.1782
Fibrin	0.1110	0.1111

The significance of the discrepancy is not apparent. It cannot be due to the partial interaction of the ω-amino group of lysine with nitrous acid and not with triketohydrindene hydrate, otherwise the same discrepancy would show itself with casein and peptone (Witte). We are forced for the present to ascribe it to some physical characteristic of the proteins mentioned. The agreement of the results of the two methods with the remaining proteins in the very early stages of hydrolysis and with the peptones makes it extremely probable that the colorimetric process estimates the amino group α to a carboxyl group, even when the latter is bound in a peptide linking, as amide nitrogen gives no ninhydrin reaction. The point, however, requires fuller experimental investigation with purified peptones and synthetic polypeptides, etc.

On comparing the three methods of following protein hydrolysis in very dilute solution, and where the results are not complicated by the presence of large amounts of ammonia, it is evident that the colorimetric or the Van Slyke methods are to be preferred. The Sörensen method, however, is much the simplest to

carry out, though its range of application does not allow it to be used in extremely dilute solutions of proteins.[7] We should, however, mention in this connection a recent paper by Clementi,[8] who, by an improved and accurately calibrated burette, has made the Sörensen method sensitive with solutions containing less than 1 mg. of amino-acid per 1 cc. The most sensitive method is undoubtedly the colorimetric method, and it is also relatively simple in manipulation. It is inapplicable, however, in strongly acid or alkaline solutions, and will probably find its greatest usefulness in the study of protein hydrolysis in neutral and faintly alkaline media. The gasometric method, owing to its susceptibility to correction, for the nitrogen of amino groups other than the α-amino group of amino-acids, is the most accurate of the three. The ninhydrin reaction is also given by a number of amines;[9] but it will be shown in a later paper from this laboratory that these are capable of classification, and fall into one or two well defined groups.

SUMMARY.

The colorimetric method for the estimation of amino-acid α-nitrogen can be used to follow the hydrolysis of proteins by pancreatic enzymes. The results agree with those obtained by the Van Slyke method.

EXPERIMENTAL.

Casein.

The casein employed in the following digestion was purified according to the method of Robertson.[10] 5.6 gm. of this pure casein were dissolved in 44.8 cc. 0.1 N NaOH in pursuance of Walters'[11] directions for preparing basic sodium caseinate (0.4 per cent). To this were added 300 cc. distilled water and, after boiling and filtering, the clear filtrate was made up to 1,400 cc.

[7] Potter, R. S., and Snyder, R. S., *J. Ind. and Eng. Chem.*, 1915, vii, 1049.

[8] Clementi, A., *Atti Accad. Lincei*, 1915, series 5, xxiv, 51, 102.

[9] Neuberg, C., *Biochem. Z.*, 1913, lvi, 500.

[10] Robertson, T. B., *J. Phys. Chem.*, 1910, xiv, 534.

[11] Walters, E. H., *J. Biol. Chem.*, 1912, xi, 269.

100 cc. protein solution, 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 1 per cent holadin solution, and 2 cc. toluene were used in each experiment.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0.5	0.0386	0.0297	0.0384
12	0.1628	0.1484	0.1638
24	0.1748	0.1696	0.1754
36	0.1991	0.1838	0.2000
48	0.2068	0.1987	0.2082
72	0.2211	0.2050	0.2180
84	0.2257	0.2121	0.2222
120	0.2329	0.2263	0.2326
156	0.2440	0.2403	0.2462
180	0.2510	0.2474	0.2564
216	0.2657	0.2615	0.2632

Serum Albumin and Globulin.

In the endeavor to prepare a 0.5 per cent solution, 6 gm. were dissolved in dilute NaCl solution and filtered. The clear filtrate was diluted to 1,200 cc. and employed as follows: 100 cc. protein solution, 1 cc. 0.5 per cent Na_2CO_3, 1 cc. pancreatic extract, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0612	0.0282	0.0200
2	0.1538	0.0991	0.1471
5.5	0.1638	0.1061	0.1622
14.5	0.1812	0.1169	0.1786
19.5	0.1841	0.1204	0.1818
24	0.1880	0.1346	0.1832
36.5	0.1880	0.1346	0.1872
48	0.1915	0.1346	0.1922
60.5	0.1922	0.1417	0.1930
84.5	0.1950	0.1417	0.1952
114.5	0.1997	0.1558	0.1976

Peptone (Witte).

A 0.5 per cent solution of peptone (Witte) was made by boiling and filtering off a slight insoluble residue. To 100 cc. portions of this solution were added: 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 0.2 per cent holadin solution, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0734	0.0707	0.0719
8	0.1511	0.1357	0.1538
20.5	0.1518	0.1640	0.1801
32	0.2165	0.1753	0.2150
44	0.2267	0.1809	0.2273
56	0.2332	0.2036	0.2296
68	0.2379	0.2036	0.2381
80	0.2396	0.2112	0.2409
92	0.2417	0.2149	0.2424
104	0.2441	0.2149	0.2439
128	0.2451	0.2205	0.2438
140	0.2467	0.2262	0.2462

Peptone e Carne.

A 0.5 per cent solution of peptone *e carne* was prepared by boiling and filtering off the slight insoluble residue. To 100 cc. portions of this solution were added: 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 0.2 per cent holadin solution, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0773	0.0707	0.0807
5	0.1232	0.1201	0.1250
29	0.1477	0.1414	0.1471
57	0.1552	0.1414	0.1666
74	0.1569	0.1414	0.1586
96	0.1694	0.1484	0.1666
120.5	0.1749		0.1754
145	0.1762	0.1520	0.1786
174	0.1860	0.1555	0.1852
218.5	0.1922	0.1555	0.1952

Nucleoprotein.

2.5 gm. nuclein (Schuchardt) were dissolved in 60 cc. 0.5 per cent Na_2CO_3 by boiling. After filtering, a residue of 0.2370 gm. remained, and the filtrate was diluted to 500 cc. 50 cc. samples were treated with 0.5 cc. pancreatic extract and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0174	0.0177	0.0176
3.5	0.1500	0.1275	0.1275
10.25	0.1895	0.1558	0.1851
15	0.2244		
25.25	0.2297	0.2125	0.2322
42.25	0.2371	0.2267	0.2388
55.25	0.2397	0.2267	0.2418

Gluten.

2.5 gm. gluten (Schuchardt) were dissolved in 60 cc. 0.5 per cent Na_2CO_3 at ordinary temperature, and, after filtering off a slight insoluble residue, made up to 500 cc. volume. 50 cc. portions of this solution were treated with 0.5 cc. pancreatic extract and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
0	0.0313	0.0283	0.0293
2	0.1091	0.0850	0.1020
5.5	0.1182	0.0855	0.1150
13	0.1536	0.1154	0.1492
21	0.1673	0.1275	0.1640
33.5	0.1803	0.1452	0.1886
46.5	0.2036	0.1558	0.1899
72	0.2260	0.1771	0.2238
93	0.2390	0.1771	0.2343
100	0.2421	0.1842	0.2418

Fibrin.

The fibrin used in this digestion was obtained from ox blood. After removing a large excess of moisture by filter paper, analysis proved the presence of 46.98 per cent H_2O. To 100 cc. portions of distilled water were added: 0.5 gm. fibrin, 1 cc. pancreatic extract, 1 cc. 0.5 per cent Na_2CO_3, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
5	0.0833	0.0495	0.0602
15	0.1110	0.0777	0.1111
27	0.1356	0.0848	0.1338
39.5	0.1389	0.0919	0.1374
51	0.1392	0.0989	0.1388
76	0.1416	0.0989	0.1408
99.5	0.1423	0.1060	0.1424
124	0.1469	0.1131	0.1444
148.5	0.1477	0.1133	0.1471

Gelatin.

6 gm. pure sheet gelatin (isinglass) were dissolved in boiling water and, after filtering, diluted to 1,200 cc. To 100 cc. portions of this solution were added: 1 cc. 0.5 per cent Na_2CO_3, 1 cc. 1 per cent holadin solution, and 2 cc. toluene.

Time.	Amino nitrogen per cc.		
	Van Slyke.	Sörensen.	Colorimeter.
hrs.	*mg.*	*mg.*	*mg.*
1	0.0352	0.0245	0.0320
4	0.0986	0.0707	0.0921
10	0.1031	0.0848	0.1021
22	0.1204	0.1060	0.1212
34	0.1373	0.1095	0.1351
46	0.1440	0.1201	0.1462
58	0.1489	0.1272	0.1497
72.5	0.1561	0.1484	0.1562
94	0.1636	0.1520	0.1614
119.5	0.1698	0.1520	0.1666
142	0.1725	0.1555	0.1708

IMPROVED GAS CHAIN METHODS OF DETERMINING HYDROGEN ION CONCENTRATION IN BLOOD.

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(From the Physiological Laboratory of the University of Minnesota, Minneapolis.)

(Received for publication, December 1, 1915.)

The Gas Chain Method for Blood.

The most accurate determinations of the hydrogen ion concentration of human blood seem to be those of Hasselbalch on defibrinated reduced blood resaturated with CO_2 at alveolar tension. The objections to his method are first, the difficulties of manipulation; second, the time required to reduce the blood; and third, the error due to the deterioration of electrode and blood during reduction.[1]

In order to avoid loss of CO_2 and of time in the reduction of blood, I devised an electrode that could be filled directly from the blood vessel.[2] But in order to determine the buffer value of blood and to avoid the use of the expensive hirudin, I have modified it as follows.

FIG. 1. Hollow needle, 7 cm. long and 0.7 mm. bore.

The blood is drawn from the vein by means of the hollow needle (Fig. 1), of 0.7 mm. bore and 7 cm. long, connected with the 12 cc. defibrinating tube (Fig. 2) by means of a short piece of strong rubber tubing, in such a manner that all of the blood that is exposed to the air overflows out of the tube. A piece of glass tubing is connected to the open end of the defibrinating tube by means of rubber tubing. When the defibrinating tube is full the two rubber tubes are closed with pinch-cocks or Langenbeck

[1] Hasselbalch, K. A., and Gammeltoft, S. A., *Biochem. Z.*, 1915, lxviii, 235, 236.

[2] McClendon, J. F., *Am. J. Physiol.*, 1915, xxxviii, 181.

clips and the blood is defibrinated with the lead ball by shaking the tube. As the blood cools and contracts the clip is opened from time to time in order to allow blood to return from the overflow tube and thus prevent the escape of gas from the blood, due to diminished pressure.

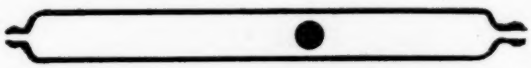


FIG. 2. 12 cc. defibrinating tube.

When defibrination is complete the closure of the rubber tubes is secured by folding them back over the nipples of the defibrinating tube and wrapping them with wire or cord. The defibrinating tube is then placed in the centrifuge, balanced with a similar tube, and the fibrin and corpuscles are precipitated. When it is removed from the centrifuge the wires are replaced by clips. The lower rubber tube is connected to a long rubber tube and funnel filled with mercury in such a manner that no air is admitted. The upper rubber tube is connected with the left end of the electrode of 5 cc. capacity shown in Fig. 3. By opening the cocks and raising the funnel, the electrode is filled with serum in such a manner that all of the serum that comes in contact with the air overflows through the stop-cock into the overflow tube.

A small bubble (0.1 cc.) of pure hydrogen (or $H_2 + CO_2$) is admitted into the electrode and both ends of the latter are closed. The electrode is shaken until the bubble comes to equilibrium with the serum as regards CO_2 tension, when the bubble is allowed to rise until it completely surrounds the platinum foil. The electrode is then set up and allowed to come to the temperature of the room. It is connected to the electrometer by means of a copper wire hook that engages a hook on the end of the platinum foil. The copper wire is long and coiled in a close spiral to reduce its stiffness, and is small enough so that its weight does not straighten out the hook on the platinum. If copper and platinum are bright and dry the contact is usually good enough to get the zero point with the electrometer, and if this is not the case it is immediately detected by failure of the electrometer to show a sharp zero point on the potentiometer wire. The overflow tube is connected with the saturated KCl solution into which the calomel electrode dips, by a piece of absorbent cord. The

stop-cock is not greased and need not be opened while taking a reading, but must be opened momentarily immediately before the reading in order to equalize the pressure of the hydrogen, which has been reduced by partial combination with oxygen dissolved in the serum.

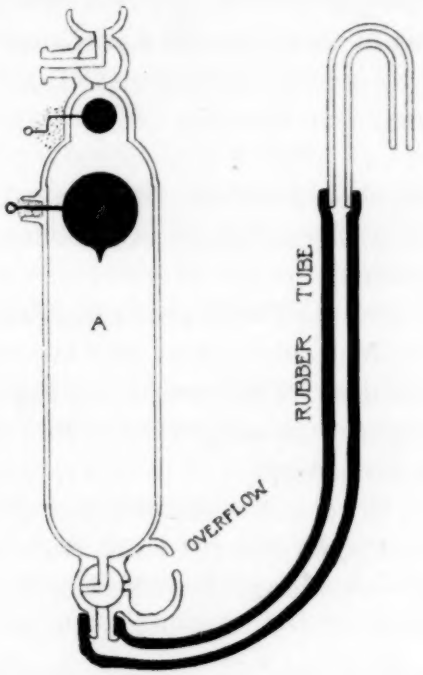


FIG. 3. Hydrogen electrode, actual size. The stop-cocks are bored at right angles so that the connecting tubes may be rinsed out through the extra openings in the stop-cocks. The lower cock is lubricated with KCl solution before filling the electrode, and its bore may be filled with the same solution and turned as in the figure if it is desired to have a wider surface of contact between this solution and the electrode contents. The two discs are gold or gold plated platinum and are platinized. In case CO_2 loss is to be avoided the upper one is used, but with known constant CO_2 pressure, the lower one is used and the H_2—CO_2 mixture forced in until only the point of the disc touches the liquid. The rubber connection filled with KCl solution allows mechanical inversion of the electrode about the axis, A, without disconnection from the potentiometer (thus replacing the absorbent cord).

A succession of readings are taken until a maximum difference of potential is obtained and remains constant 5 minutes, which denotes that all of the oxygen contained in the hydrogen has disappeared. If it is not certain that CO_2 equilibrium was established the electrode must be shaken again, after which some time

will be required to reduce the additional oxygen that has been brought up from the lower part of the electrode. Usually 200 inversions of the electrode are sufficient for CO_2 equilibrium and 10 minutes sufficient for disappearance of the oxygen.

After the above procedure the serum has the same hydrogen ion concentration as the blood in the vein except for temperature change and the loss of CO_2 into the hydrogen. By reducing the ratio of hydrogen to serum the loss of CO_2 may be made small. It may be still further reduced by using hydrogen containing 5 per cent CO_2.

The platinum foil should be 0.02–0.05 mm. thick, or if a wire is used, the part inside the electrode vessel should be beaten thin and thoroughly cleaned.

The electrodes are cleaned with potassium bichromate in H_2SO_4, are replatinized for about 30 seconds, and are tested with a standard phosphate mixture. Ten freshly platinized electrodes are tested with it and unless the majority of them agree to a millivolt the determination is repeated.

The rubber tube for the defibrination apparatus must be very strong and renewed each time. It is well to attach it with rubber cement and wire before the experiment.

The Determination of the CO_2 Pressure on the Same Sample.

Having once determined the C_H of the serum, which is the same as that of the blood in the vein, the CO_2 pressure of the sample may be approximated by exposing it to a series of CO_2 pressures and noting under which the C_H is nearest to the original value.

The CO_2 and H_2O are mixed in a tonometer. For this purpose the tonometer is made like a Hempel burette, except that one end is wide and the other constricted and finely graduated from 0 to 10 cc. The tonometer is set up vertically with a mercury funnel and a 1 cm. vertical glass tube used as a sight in leveling the mercury surfaces. When the mercury in the funnel, tonometer, and 1 cm. tube is on a level the amount of gas introduced may be read on the scale. The tubes leading from the CO_2 and H_2 generators are connected with side tubes several meters long, filled with the respective gases in order to maintain them at atmospheric pressure while being introduced into the tonometer.

The serum is transferred to the tonometer and rotated 15 minutes by a rubber band that passes over it and an axle. The serum is returned to the electrode, a bubble of the same gas mixture added, and the reading taken. If care is taken in this manipulation, no oxygen is admitted and equilibrium is attained more quickly than for the first reading, sometimes in 2 minutes. The next lower per cent CO_2 is now used, and so through the series. The results may be compared with the alveolar CO_2 pressure.

Knowing the alveolar CO_2 tension, one can determine the P_H of arterial blood. We find it the same as venous blood within the limits of error of a single determination (without averaging duplicates). By making both electrodes the same size, duplicate determinations may be made in the same electrode vessel. If the volume of pure hydrogen is increased to 1 cc. the maximum error is 1 millivolt.

It is claimed by Hasselbalch that serum is more alkaline than blood. Hasselbalch admits that adding oxygen to blood makes it read less alkaline. Evidently oxygen acts by combining with the hydrogen in the platinum black. Serum reads more alkaline than blood because it contains less oxygen, but the reading is more nearly the true reaction of the blood. In order to decrease the danger of introducing oxygen the electrode is filled with hydrogen before use.

The Buffer Value of Serum.

Since the buffer value of a fluid means its resistance to change of reaction by the addition of acids or bases, the change of reaction on the addition or removal of CO_2 gives an index of the buffer value. It is only necessary to determine the C_H of the serum at two known CO_2 pressures to obtain an index of the buffer value. It would be convenient to have some standard of pressure, and 10 per cent and 3 per cent of an atmosphere are suggested. If the CO_2 pressure of the serum has been determined by the above method, these data are already at hand; if not, the rule should be always to begin with the higher pressure.

Thermoregulation.

Owing to the inconvenience of small thermostats, the laboratory was kept within 0.3° of 22° by means of an electric fan and the apparatus shown in Fig. 4. The essentials of a good system are low heat capacity of regulator and heater and rapid conduction. The regulator is made of a strip of invar and brass welded together (combined thickness, 1 mm.). The heater is bare nichrome wire strung through the air.

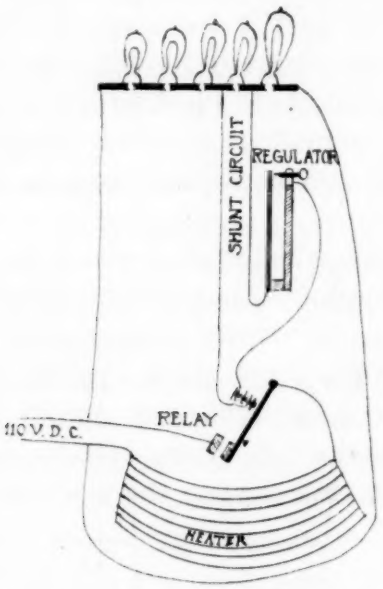


FIG. 4. Thermoregulator.

Table for Converting Millivolts to Hydrogen Ion Concentration.

Since the table by Schmidt[3] is for only one temperature, 18°, the range of 18–30° was selected in preparing the graph in Fig. 5. In 1908 the International Electrical Congress established the international ohm and fixed the ampere as the current that deposits 0.001118 gm. Ag per second. Taking the atomic weight of Ag as 107.88, the Faraday or electrical equivalent is 96,494 coulombs. The value 96,500 has come into use. If these new data are used exclusively, the formula for C_H is $P_H = -\log C_H = \frac{\text{E. M. F.}}{0.0001984\ T}$

[3] Schmidt, C. L. A., *Univ. California Publications, Physiology*, 1905–10, iii, 104.

which is nearly the same as the old formula. The value of the volt was still uncertain, however, until the International Committee, at Washington, 1910, standardized the method of washing the cathode of the silver coulometer, and fixed the E.M.F. of the International Standard Weston Cell at 1.0183 at 20°. According to Rosa,[4] it is more nearly 1.01827, but this difference is less than the individual variations of cells. It was pointed out by Wolff[5] that no exactly correct formula for the temperature effect on the E.M.F. has been devised, and that from 2 to 24 hours are necessary for equilibrium after an abrupt change of temperature. From Wolff's formula, which is more accurate than previous formulas, it may be seen that the change in E.M.F. per degree is more nearly 0.04 millivolt than 0.038 as given by Jaeger and Wachsmuth. Where temperature control is not adequate, it might seem safer to use a cell from the Weston Company, saturated at 4°, E.M.F. = 1.0186, independent of temperature.

There is considerable difference in the correction for calomel electrodes as applied by different writers. G. N. Lewis[6] uses a value 8 millivolts different from that used by other investigators for the normal calomel electrode. He bases this value on a calculation of the dissociation of the acid used in the normal hydrogen electrode from thermodynamical data, which makes the dissociation of strong acids more nearly obey the law of mass action. Since so much work is based on the calculation of C_H from electrical conductivity, it might seem safer at present to continue this practice.

Although the saturated KCl calomel electrode of Michaelis[7] is the most convenient, if work is done at various temperatures the 0.1 N KCl calomel electrode is better on account of the lower temperature coefficient. Since there is a variation in the value of the correction for the 0.1 N electrode used by different writers, it is hoped that some standard value may be decided on. Ostwald[8]

[4] Rosa, E. B., Vinal, G. W., and McDaniel, A. S., *Bull. of the Bureau of Standards, U. S. Dept. of Commerce*, 1913, ix, 493.

[5] Wolff, F. A., *Bull. of the Bureau of Standards, U. S. Dept. of Commerce*, 1908, v, 309.

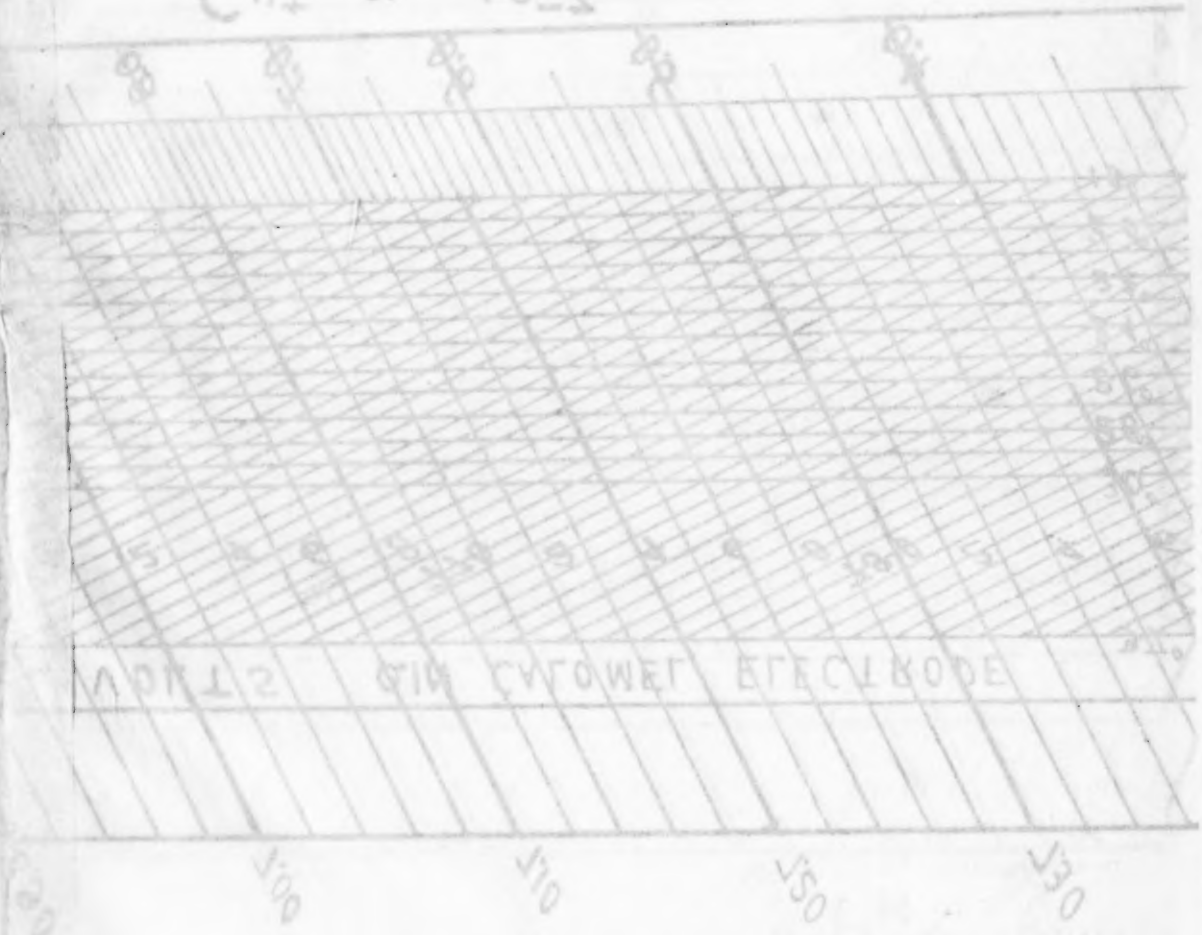
[6] Lewis, G. N., and Randall, M., *J. Am. Chem. Soc.*, 1914, xxxvi, 1969.

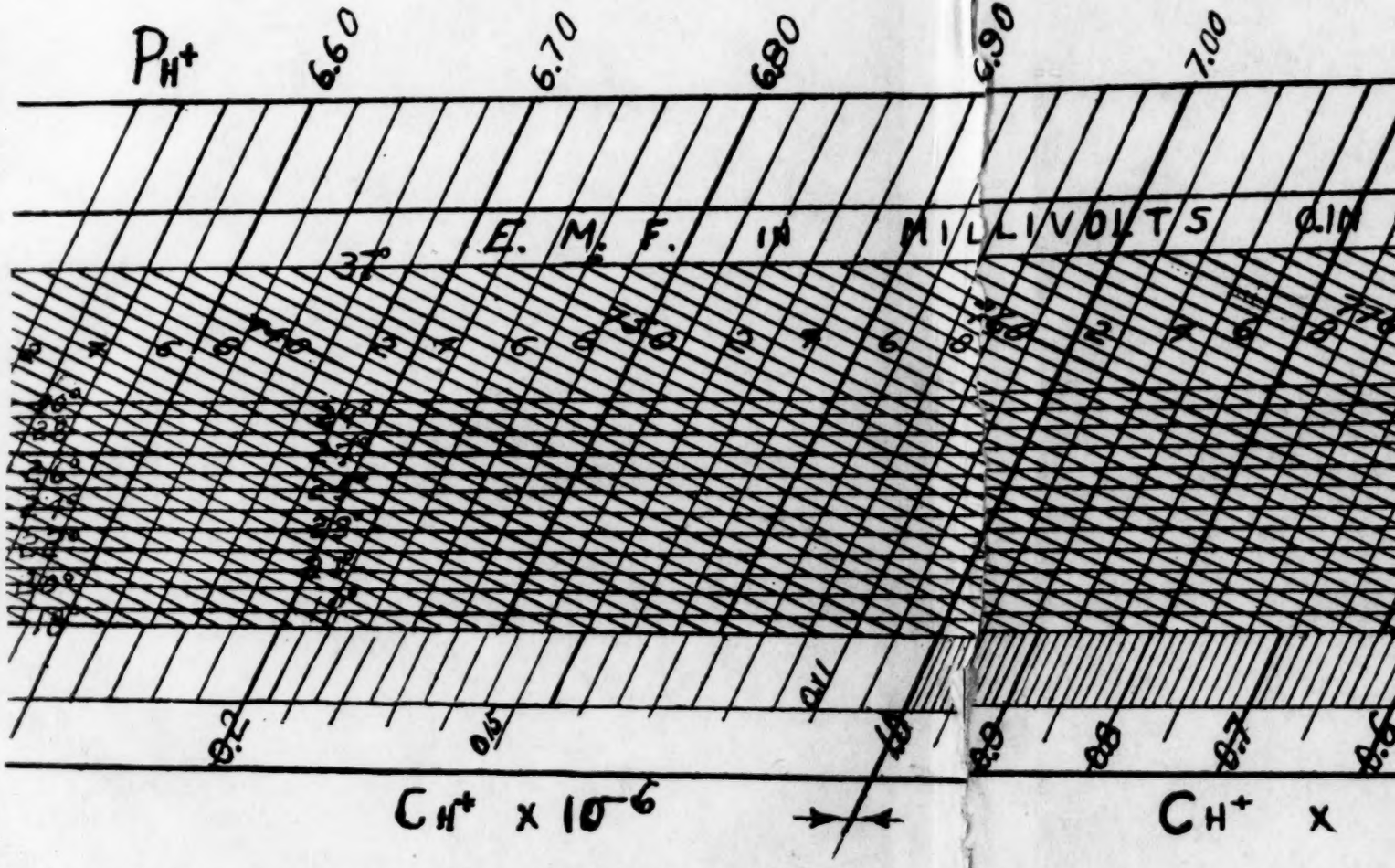
[7] Michaelis, L., Die Wasserstoffionenkonzentration, Berlin, 1914.

[8] Ostwald, W., and Luther, R., Physiko-chemische Messungen, Leipsic, 3rd edition, 1910.

found no difference due to size of grain of calomel, since samples of calomel contain grains of various sizes, and the only variation is due to impurity of ingredients or incorrect concentration of KCl, which can easily be avoided. It is well to seal the electrode in a flame and have the tip of the syphon always closed by a non-greased stop-cock or ground cap which is immersed in a closed vessel of 0.1 N KCl solution; a second syphon connects with saturated KCl. Auerbach,[9] who corrects for the water vapor in the normal hydrogen electrode, found the E.M.F. of this when coupled with the 0.1 N KCl calomel electrode to be 0.337 at all temperatures from 0–30°, and it would be convenient if this value were accepted generally. Our formula then becomes, $P_H = -\log C_H = \frac{\text{E. M. F.} - 0.337}{0.0001984\ T}$. The partial pressure of hydrogen in the hydrogen electrode is reduced by [(760 − barometric pressure) + (mm. CO_2) + (mm. N_2) + (vapor pressure)] and may be made equal to 760 mm. by applying pressure to the overflow tube of the hydrogen electrode and closing the stop-cock, or the reading may be corrected by adding to it $\frac{0.0001984\ T}{2} \log q$ where q is the partial pressure of hydrogen in the electrode. The nitrogen pressure may be taken at 10 mm. and the vapor pressure is practically that of pure water. The correction for blood is about 1.7 mv. with the barometer at 740.

[9] Auerbach, F., *Z. Elektrochem.*, 1912, xviii, 13.




PH+
6.60
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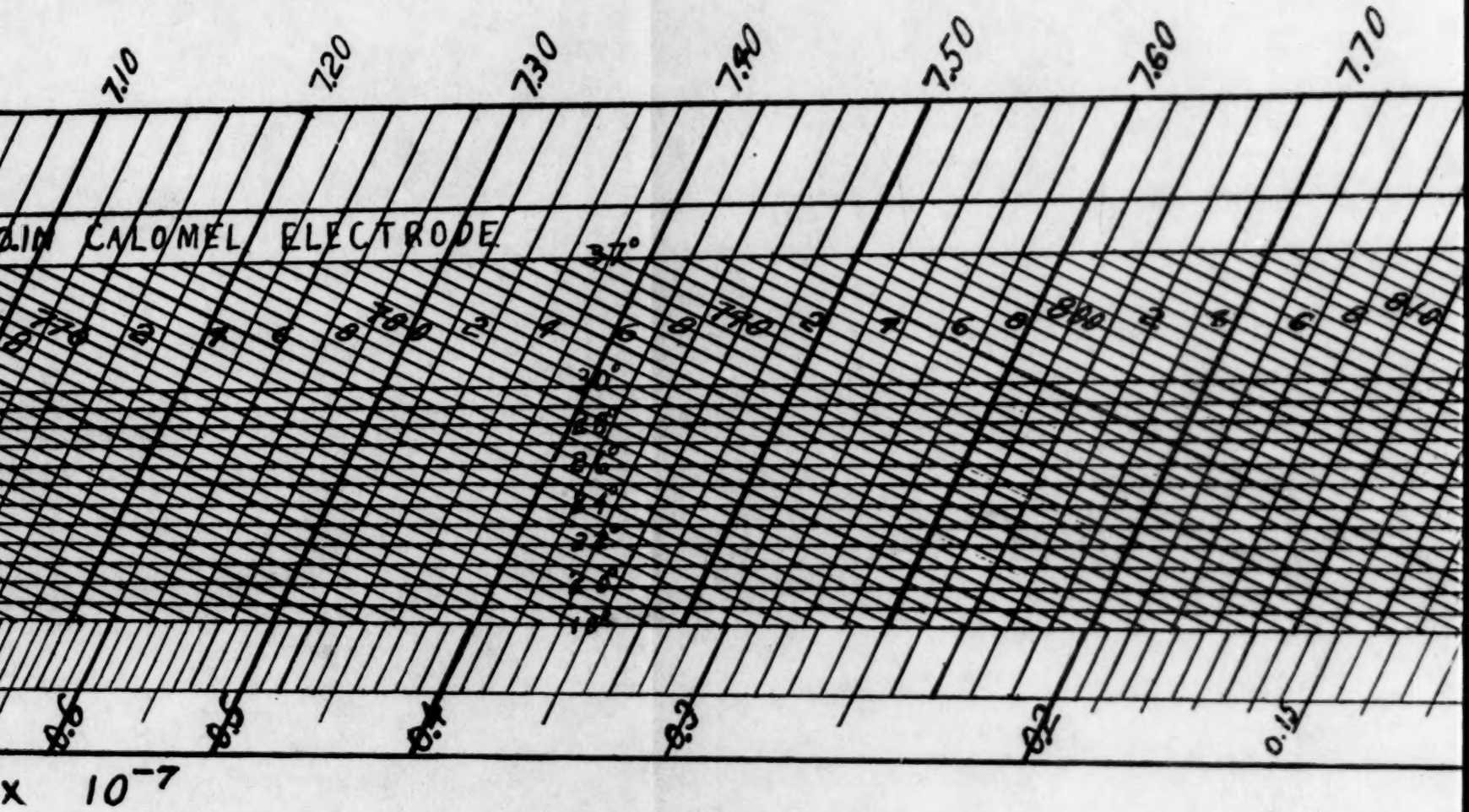


FIG. 5. Table for converting millivolts into P_H and C_H, using 0.1 N KCl calomel electrode.

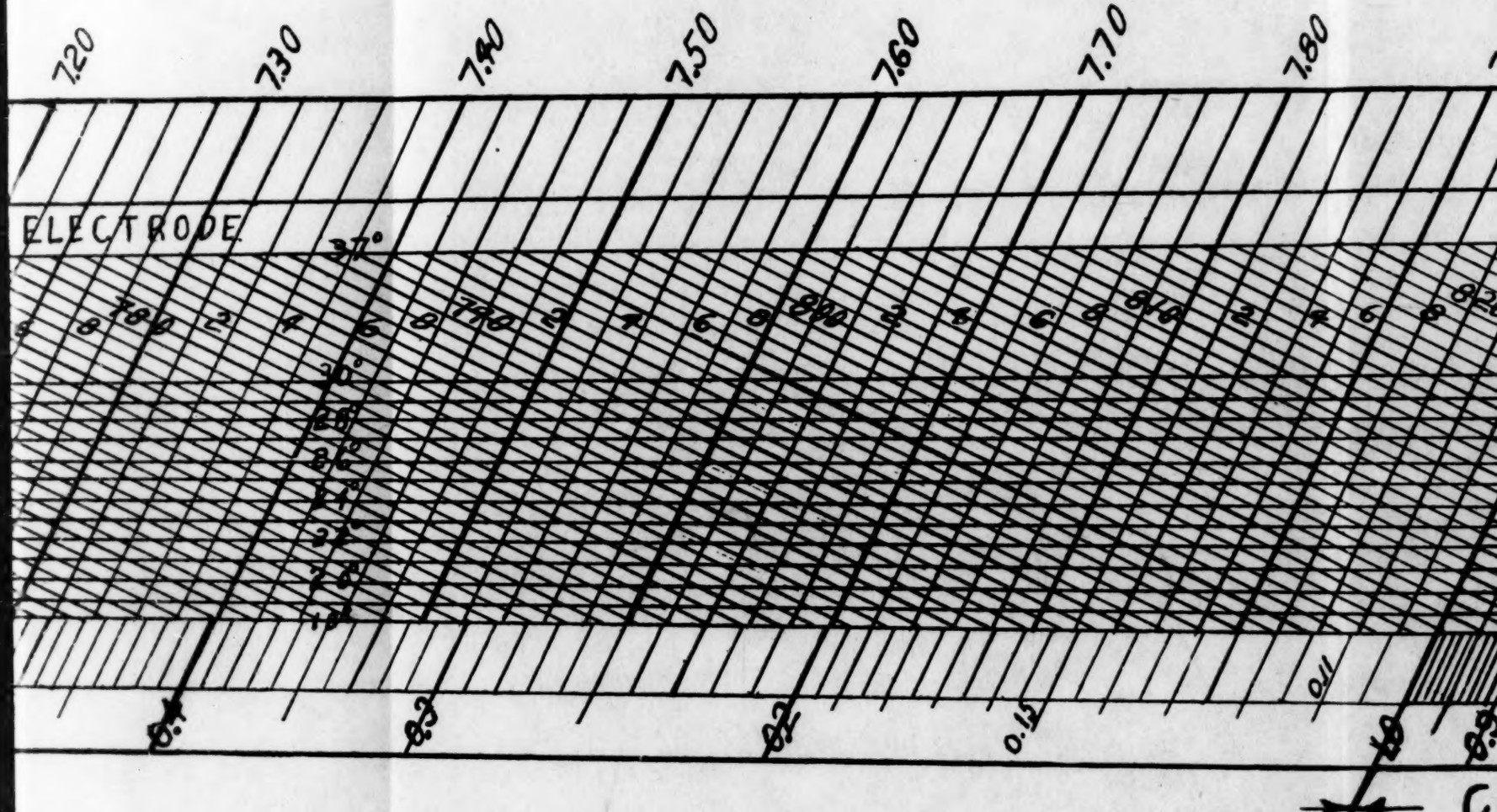
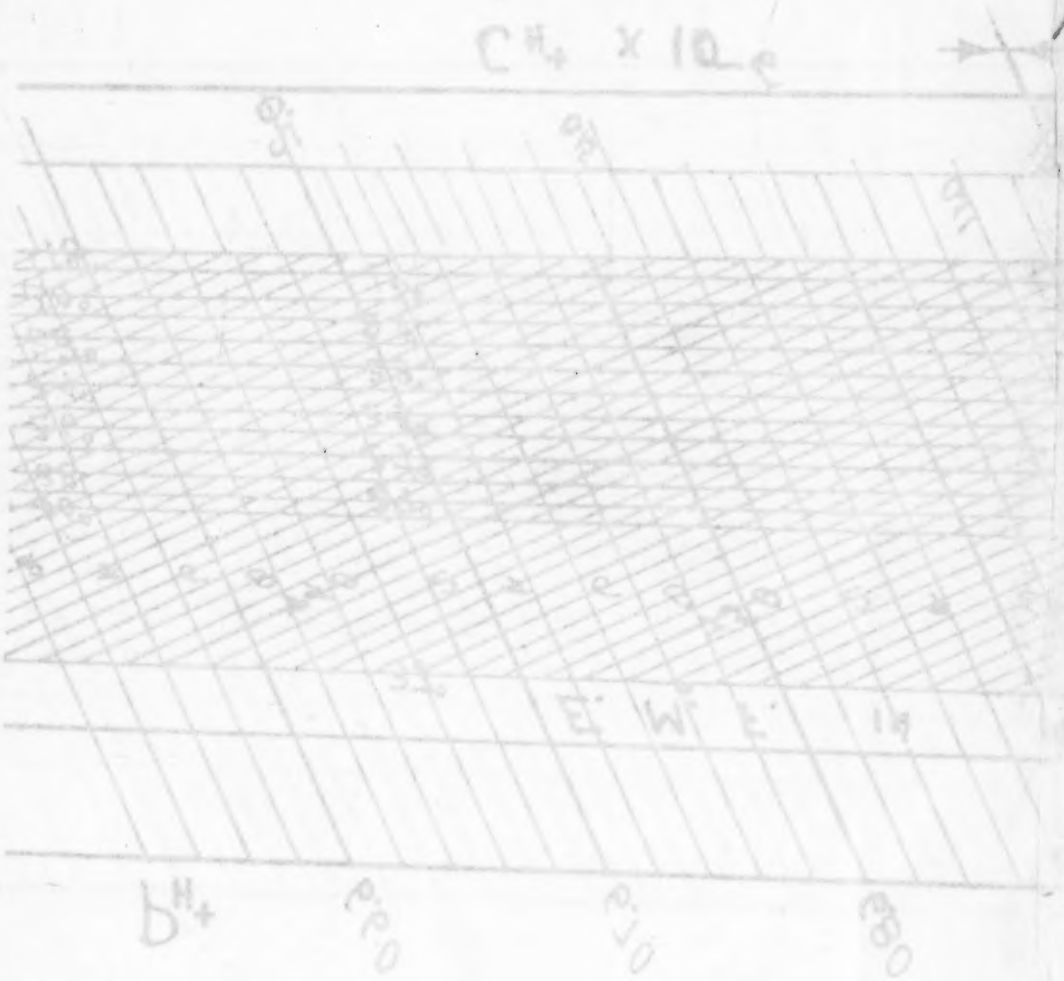


FIG. 5. Table for converting millivolts into P_H and C_H, using 0.1 N KCl calomel electrode.

7.90
8.00
8.10
P_{H^+}
8.20
8.30
8.40
37
8.20
8.40
0.9
0.8
0.7
0.6
0.5
0.4
0.3
$C_{H^+} \times 10^{-8}$



SOME COLOR REACTIONS FOR INDOLE AND SCATOLE.*

By V. E. NELSON.

(*From the Laboratory of Agricultural Chemistry of the University of Wisconsin, Madison.*)

(Received for publication, February 24, 1916.)

In an effort to secure a more rapid and accurate separation and estimation of indole and scatole in fermentation mixtures certain substances were found to give characteristic color reactions with one or the other of these two compounds.

Dimethylaniline when added to a solution of scatole in the presence of sufficient sulfuric acid produces a deep red-violet coloration which is completely soluble in chloroform. A few drops of dimethylaniline are thoroughly emulsified with 5 or 6 cc. of scatole solution in a test-tube and concentrated sulfuric acid is added cautiously down the side of the tube so as to form a layer at the bottom. A beautiful violet ring forms at the junction of the two liquids which is still visible at a dilution of one part in 1,000,000. Indole produces a faint red under similar conditions, but the color is not soluble in chloroform. When sulfuric acid is replaced by either hydrochloric or phosphoric acids no color is developed. The sulfuric acid used was boiled so as to free it from nitrous acid which invariably accompanies most of the so called chemically pure acid. Dimethylaniline together with concentrated sulfuric acid produces no color, and scatole and sulfuric acid develop only a faint pink coloration.

Pyruvic aldehyde gives with indole, sulfuric acid, and a crystal of ferric sulfate a deep red-violet color which cannot be extracted by chloroform, carbon bisulfide, or amyl acetate, but is soluble in amyl alcohol. If a few crystals of pyruvic aldehyde are added to 5 or 6 cc. of an indole solution containing a small crystal of ferric sulfate and sulfuric acid added cautiously so as to form a

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layer at the bottom of the tube a red-violet ring forms. The test is sensitive to about one part in 500,000. A solution of scatole when subjected to similar treatment gives a yellow coloration soluble in amyl acetate.

When a few crystals of glycolic acid are dissolved in a solution of scatole, and sulfuric acid equivalent to the volume of the solution is added, a beautiful deep red-violet color is formed. A trace of ferric sulfate intensifies the reaction. The color is completely soluble in chloroform, but only a slight brownish tint is extracted by amyl acetate. Indole with glycolic and sulfuric acids gives a brown coloration which is not soluble in chloroform. The test is easily sensitive to one part in 1,000,000.

A drop or two of a solution of glyceric aldehyde when added to a solution of scatole in the presence of sulfuric acid produces an intense red color completely soluble in chloroform. Indole under similar conditions gives a yellow color insoluble in chloroform.

If to 5 or 6 cc. of an indole solution there are added a few drops of a 5 per cent solution of vanillin in 95 per cent alcohol and the mixture is made strongly acid with 3 or 4 cc. of concentrated hydrochloric acid a beautiful orange coloration forms. A solution of scatole to which vanillin and hydrochloric acid are added gives a deep violet color upon heating. When sulfuric acid is substituted for hydrochloric acid the colors developed by indole and scatole are orange and deep red to violet respectively. Phosphoric acid and vanillin give a deep violet with scatole and an orange coloration with indole. If the scatole coloration is extracted with chloroform it changes to a light orange. Vanillin and hydrobromic or acetic acids give no color with indole or scatole. If hydrofluoric acid is substituted for the hydrobromic acid then a deep violet color forms with scatole and an orange color with indole. The violet upon extraction with chloroform changes to a deep orange.

The exact nature of the compound formed with vanillin is not known. It would appear from the solubility and changes of color in chloroform that the scatole compound bears some relation to the acid used to produce it. The indole compound seems to be the same regardless of the acid employed.

The vanillin method of detecting indole and scatole is a very sensitive reaction. Scatole, vanillin, and hydrochloric acid give

a distinct deep violet at a dilution of one part in 2,000,000 and the color is rendered more perceptible by shaking with a few drops of chloroform. When hydrofluoric or sulfuric acids are used the test is sensitive to dilutions of one part in 2,000,000 and 3,000,000 parts respectively.

Phosphoric acid gives with vanillin and scatole a violet color up to dilutions of about one part in 2,000,000. Indole, vanillin, and hydrochloric or phosphoric acids give a test up to dilutions of one part in 5,000,000 and with sulfuric acid the color is easily discernible in a strength of one part in 2,000,000.

Steensma[1] maintained that the colors produced with indole and scatole by vanillin and hydrochloric acid could not be used as a means of their identification in a mixture of these two substances.

Because of the sensitiveness of the reaction and the distinct colors produced with these compounds and vanillin it was thought that if a solvent could be found which would extract one of the colors without extracting the other it would render the test useful qualitatively in a mixture and perhaps quantitatively also.

The following table gives the solubility in different solvents of the colors developed by indole and scatole together with vanillin, and hydrochloric, sulfuric, phosphoric, and hydrofluoric acids. Only a few of the many solvents used are given in the table.

It is to be noted that the scatole coloration is soluble in chloroform, amyl acetate, and amyl valerianate while the color produced by indole is very slightly soluble in these reagents. It is, therefore, possible to apply the test satisfactorily to a mixture of indole and scatole.

In the case of a mixture of the two compounds a few drops of a 5 per cent solution of vanillin in 95 per cent alcohol are added and sufficient acid to obtain a strong development of color. The solution is brought to a boil, cooled, and amyl acetate, amyl valerianate, or chloroform added, and shaken a few times. If hydrochloric or sulfuric acids are used the extraction of a deep violet indicates the presence of scatole. With phosphoric or hydrofluoric an orange to red color is extracted if scatole is present. With the last two acids the solution must stand for several minutes after boiling, before the maximum color develops.

[1] Steensma, F. A., *Z. physiol. Chem.*, 1906, xlvii, 25.

TABLE I.

Solubility in Different Solvents of Colors Developed by Scatole and Indole with Vanillin and Various Acids.

Color.	Chloroform.	Amyl acetate.	Carbon disulfide.	Benzaldehyde.	Amyl valerianate.	Amyl alcohol.
Deep violet produced by boiling scatole, vanillin, and HCl.	Readily soluble.	Soluble.	Insoluble.	Soluble.	Soluble.	Soluble.
Deep red to violet produced by scatole, vanillin, and H_2SO_4.	Soluble.	"	"	"	"	Soluble but color changes to an intense blue.
Deep violet produced by boiling scatole, vanillin, and H_3PO_4.	Soluble but color changes to an orange.	Soluble but color changes to red.	"	"	Sparingly soluble with change of color to orange.	Soluble.
Deep violet produced by scatole, vanillin, and HF, on boiling and allowing to stand a few minutes.	Soluble but color changes to a deep orange.	—	—	—	—	—
Orange color produced by indole, vanillin, and HCl.	Very slightly soluble.	Insoluble.	Insoluble.	Soluble.	Insoluble.	Soluble.
Orange produced by indole, vanillin, and H_2SO_4.	Insoluble.	"	"	"	"	"
Orange developed by indole, vanillin, and H_3PO_4.	"	"	"	"	"	"

As amyl acetate, amyl valerianate, and even chloroform effect such a good separation of the colors produced by scatole and indole with vanillin and hydrochloric acid it was thought that this method might be applied quantitatively. A large number of determinations using known amounts of scatole were made and the majority of the results agreed very closely, but now and then for

some reason a large divergence was noted so that further work upon this will have to be done before the method can be pronounced satisfactory.

It might be mentioned that tryptophane also gives a coloration varying from a deep red to a deep violet with vanillin and sulfuric acid. The color is not soluble in chloroform and the reaction is not nearly so sensitive as with indole and scatole.

If to a little casein in a test-tube are added a little water, a few drops of a 5 per cent solution of vanillin in 95 per cent alcohol, and sulfuric acid, a deep violet color forms. Gelatin under similar conditions does not give this coloration.

When amyl alcohol was used to extract the color produced by scatole, vanillin, and sulfuric acid, it was noticed that the solution always becomes a distinct blue in color. It was found that amyl, butyl, and propyl alcohols together with vanillin and sulfuric acid give a very intense purple coloration which changes to a deep blue upon standing a few minutes. Methyl and ethyl alcohols do not do this. When vanillin is dissolved in amyl alcohol and concentrated HCl is added, a beautiful deep green to deep blue color develops upon heating. In propyl alcohol with the aid of hydrochloric acid and heat no color results, but after standing several minutes a light green develops. Methyl and ethyl alcohols give no color under these conditions. The compound cumarin which is so closely associated with vanillin in food analysis gives no such coloration. Phosphoric acid also gives a deep blue coloration with vanillin dissolved in amyl alcohol upon heating, but gives no color when isobutyl, propyl, ethyl, or methyl alcohols are used. When vanillin is dissolved in acetone a beautiful deep blue coloration is produced upon the addition of concentrated hydrochloric acid. Cumarin under the same conditions yields a yellow-brown color.

When xylene is added to a solution containing vanillin and concentrated hydrochloric acid a beautiful rose-red coloration goes into the acid layer upon shaking thoroughly. It would seem as though the xylene contains some substance which is able to react with the vanillin in the presence of hydrochloric acid.

SUMMARY.

1. Dimethylaniline, glycolic acid, and glyceric aldehyde give characteristic color reactions with scatole and sulfuric acid.

2. Pyruvic aldehyde gives with indole, sulfuric acid, and a crystal of ferric sulfate a deep red-violet color.

3. Vanillin produces with scatole and hydrochloric acid an intense deep violet color; with indole and hydrochloric acid, an orange coloration.

4. Vanillin gives with scatole and sulfuric acid a deep red to violet color; with indole and sulfuric acid an orange color.

5. Scatole, vanillin, and phosphoric acid give a deep violet, while indole under like conditions gives an orange coloration. When hydrofluoric acid is substituted for phosphoric acid a deep violet is produced with scatole and an orange coloration with indole.

6. From the solubility tests it appears that the compound formed with scatole, vanillin, and various acids bears some relation to the acids used to produce it. The compound with indole is the same regardless of the acid employed.

7. By using chloroform, amyl acetate, or amyl valerianate it is possible to apply the vanillin method qualitatively in the case of a mixture of indole and scatole.

8. The intensity of the color produced by scatole, vanillin, and hydrochloric acid seems to be proportional to the concentration of scatole, but further work must be done before the method can be made quantitative.

9. Vanillin in amyl, butyl, or propyl alcohols gives characteristic color reactions with hydrochloric, sulfuric, or phosphoric acids. Vanillin in acetone gives a deep blue coloration upon the addition of hydrochloric acid. A rose-red is developed when xylene, vanillin, and hydrochloric acid are thoroughly shaken together.

10. A few drops of a 5 per cent solution of vanillin in 95 per cent alcohol when added to a solution of tryptophane in the presence of sufficient sulfuric acid produce a deep red to violet coloration.

INDOLE IN CHEESE.*

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(Received for publication, February 24, 1916.)

In attempting to isolate certain amines and other decomposition products resulting from the action of various microorganisms upon amino-acids found in cheese it was observed that limburger and *Handkäse* contain a considerable amount of indole and that the former also contains an appreciable quantity of phenolic bodies. This led to a study of other varieties of both soft and hard cheese to determine whether they contained such substances as indole, scatole, and phenol.

The method of procedure was to macerate about 400 gm. of cheese with water, place the mixture in a 5 liter flask, and distil with steam. The distillate might contain indole, scatole, phenol, and ammonia. The phenol was separated from the remaining substances by making the distillate alkaline with NaOH and redistilling. The ammonia was removed by rendering the last distillate acid with dilute sulfuric acid and redistilling. The tests applied for the detection of indole were: Herter's β-naphthaquinone reaction, Konto's test, cholera red reaction, Legal's reaction, pine wood test, pyruvic aldehyde reaction, nitroso-indole nitrate, and vanillin tests. For scatole the following tests were employed —the dimethylaniline test, Herter's *p*-dimethylamino benzaldehyde reaction, and the glycolic acid and vanillin tests. For the detection of phenol, Millon's reagent was used.

It will be observed in the table that of all the different cheeses examined only three varieties, namely, limburger, *Handkäse*, and camembert, contain any of these putrefactive substances. Furthermore, no scatole accompanies the indole and phenol. What-

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ever organism in cheese is concerned in the transformation of tryptophane into indole does so without the production of scatole. Camembert cheese contains considerably less indole than limburger or *Handkäse*. In fact in the cheese examined which was imported from Germany not more than traces could be detected. No scatole or phenol was present in this type of cheese.

Cheese.	Indole.	Scatole.	Phenol.
Limburger	Present.	None.	Present.
Handkäse	"	"	None.
Cheddar	None.	"	"
Swiss	"	"	"
Gammalost	"	"	"
Brick	"	"	"
Roquefort	"	"	"
Camembert	Trace.	"	"

Fifteen different limburger cheeses were examined and in no case was indole found to be absent. In a few very young samples which had not ripened sufficiently it was present in very small amounts. Scatole was always absent. The *Handkäse* used was not sufficiently ripe to say absolutely that no scatole or phenol occur in this type of cheese. The two samples examined gave distinct tests for indole, but not for scatole or phenol. We were unable to obtain further samples because of the scarcity of the cheese in this vicinity.

In order to get some idea as to the amount of indole found in a good ripe limburger cheese quantitative estimations were made according to Herter's method[1] as follows:

A 100 gm. sample of limburger cheese was macerated with water and after rendering the mixture alkaline with potassium hydroxide it was steam distilled. The distillate contained ammonia and indole, the phenol being held back by the caustic potash. The ammonia was removed by redistilling from a dilute sulfuric acid solution. The distillation was continued until no coloration was given by vanillin and sulfuric acid. To the distillate made alkaline with sodium hydroxide was added a slight excess of a 2 per cent solution of sodium naphthaquinone monosulfonate and

[1] Herter, C. A., and Foster, M. L., *J. Biol. Chem.*, 1905–06, i, 257.

the solution was allowed to stand for at least 10 minutes. The blue color which developed was shaken out completely by chloroform and the color compared in a Duboscq colorimeter with a known amount of indole dissolved in water and treated precisely in the same manner. The amount of indole found in various cheeses depended upon how far the ripening process had proceeded. Some of the young cheese contained such small amounts of indole that it was impossible to determine it quantitatively. Others contained indole to the extent of one part in 500,000 while the highest amount found was one part of indole in 52,800 parts of cheese.

Work was begun to determine what organisms are responsible for the production of indole in limburger cheese. Culture media containing tryptophane were inoculated with the three main types of organisms found in cheese; namely, the lactic, bulgaricus, and coccus forms. The composition of the medium was as follows:

	gm.
Tryptophane	0.1176
Lactose	4.0
K_2HPO_4	0.3
$MgSO_4 . 7H_2O$	0.1
NaCl	0.1
$CaCO_3$	2.0
$FeSO_4$	Trace.
Tap water	1,000 cc.

After standing for several months the flasks were opened and examined for indole, scatole, and phenol. None of these substances could be detected in the lactic and bulgaricus cultures. In the case of the coccus organism traces of indole were formed. This organism which had considerable liquefying power was isolated from a cheddar cheese and the surprising thing is that no indole is found in this type of cheese. Only one of the media was inoculated with the coccus organism. If the experiments which are now in progress prove that indole is formed by this type of organism then the explanation why no indole is found in cheddar cheese may be that the conditions prevailing are unfavorable for the growth of this organism. It is possible that the growth of the lactic and bulgaricus organisms is not vigorous enough in this kind of a medium to effectively attack the amino-acid. To obviate this it may be necessary to add to the culture a little peptone

or milk, which ought to increase the activity of the organisms without impairing the results. Until this has been done it will be impossible to say that the lactic and bulgaricus organisms do not produce these putrefactive substances. Work along this line is now in progress and will be reported later.

SUMMARY.

1. Indole and phenol were found to be present in limburger cheese.

2. Scatole was not found in limburger cheese.

3. Indole is present in *Handkäse.* It is doubtful if scatole and phenol are to be found in this type of cheese.

4. A trace of indole is present in camembert cheese. Scatole and phenol are absent in this type of cheese.

5. Cheddar, Swiss, *Gammalost,* brick, and roquefort do not contain any indole, scatole, or phenolic bodies.

6. The amount of indole in a limburger cheese naturally varies, depending upon how far the ripening process has gone. A young cheese may contain such a small amount that a quantitative estimation is impossible while a good ripe limburger cheese may contain as much as one part in 52,800 parts of cheese.

7. Lactic and bulgaricus forms of organisms when grown upon media containing tryptophane produced no indole or scatole. As growing organisms upon pure amino-acids is more difficult than upon proteins it may be necessary to add a little peptone or milk to the culture and until further work has been done upon this phase it will be impossible to say that these organisms do not produce these putrefactive substances.

8. The liquefying coccus isolated from a cheddar cheese appears to produce traces of indole from tryptophane. If the experiments now in progress confirm this statement, then the explanation why no indole is produced by this type of organism in cheddar cheese must be that conditions are unfavorable and growth is suppressed.

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